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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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X *Additional inventors are being named on the* 1 *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

METHODS FOR MAKING SIMVASTATIN AND INTERMEDIATES

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

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[ ] Application Data Sheet. See 37 CFR 1.76 (3)

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

[x] Applicant claims small entity status. See 37 CFR 1.27.

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[x] No [ ] Yes, the name of the U.S. Government agency and the Government contract number are:

Respectfully submitted,

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Dated: 2/4/04 Signature: Jeanne Amour Rice (Jeanne Amour-Rice)

PROVISIONAL APPLICATION FOR UNITED STATES PATENT

under 37 CFR §1.53(c)

for

METHODS FOR MAKING SIMVASTATIN AND INTERMEDIATES

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METHODS FOR MAKING SIMVASTATIN AND INTERMEDIATES

TECHNICAL FIELD

This invention generally relates to the field of synthetic organic and medicinal chemistry. In one aspect, the invention provides synthetic chemical and chemoenzymatic methods of producing simvastatin and various intermediates and related compounds. In one aspect, enzymes such as hydrolases, e.g., esterases, are used in the methods of the invention.

BACKGROUND

Simvastatin is a potent antihypercholesterolemic agent that is presently marketed under the name ZOCOR® (Merck). Simvastatin, Mevastatin, Lovastatin and Pravastatin are hexahydronaphthalene derivatives used as inhibitors of the enzyme HMG-CoA reductase, the rate-controlling enzyme in the biosynthetic pathway for formation of cholesterol in the human body. After oral ingestion, simvastatin, which is an inactive lactone, is hydrolyzed to the corresponding β-hydroxyacid form. This is an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase. This enzyme catalyzes the conversion of HMG-CoA to mevalonate, which is an early and rate-limiting step in the biosynthesis of cholesterol.

Mevastatin, Lovastatin and Pravastatin are natural fermentation products which possess a 2-methylbutyrate side chain at C-8 of their hexahydronaphthalene ring system. Simvastatin can be derived synthetically from a fermentation product of *Aspergillus terreus*.

Compounds possessing a C-8 2,2-dimethylbutyrate side chain, including Simvastatin, can be better inhibitors of HMG-CoA reductase than their 2-methylbutyrate counterparts. Thus 2,2-dimethylbutyrate derivatives may have greater promise for the treatment of atherosclerosis, hyperlipemia, familial hypercholesterolemia and similar disorders. However, these derivatives, including Simvastatin, are not naturally occurring and have to be produced synthetically. As a result, the introduction on the market of the more potent HMG-CoA reductase inhibitor Simvastatin has prompted the need for efficient, high yielding processes for manufacturing it.

SUMMARY

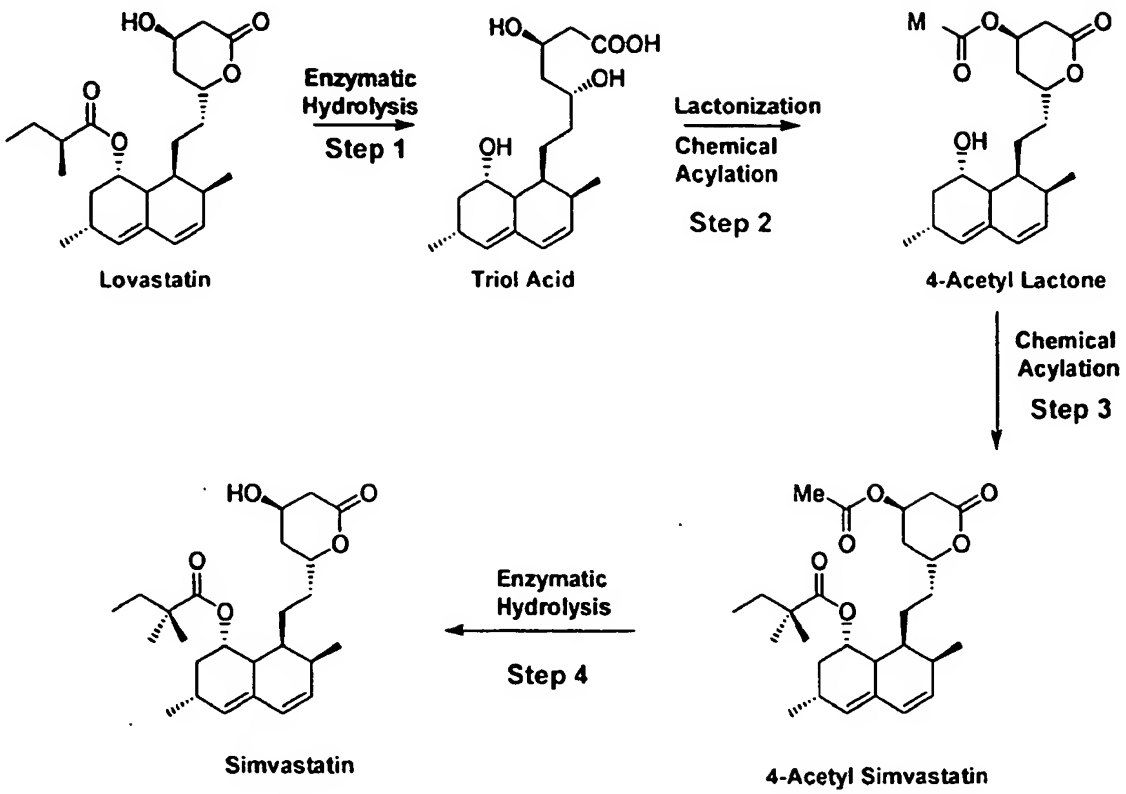
The invention provides methods for the preparation of Simvastatin, including at least one method as set forth in Appendix A or Appendix B. In one aspect, diol lactone is regioselectively acylated at the 8-position using a derivative of dimethylbutyric acid and a Lewis acid catalyst.

In one aspect, the invention provides a novel process comprising (i) the use of an enzyme of the invention (e.g., exemplary enzyme having a sequence as set forth in SEQ ID NO:4, encoded by SEQ ID NO:3) to remove the lovastatin side-chain under mild conditions, (ii) the use of the same enzyme to selectively remove an ester protecting group in the final step, and (iii) the application of novel conditions for the introduction of the simvastatin side-chain.

In one aspect, the processes of the invention generate simvastatin with <1% lovastatin present, since, in some circumstances, the separation of lovastatin from simvastatin is inefficient. In one aspect, the processes of the invention generate simvastatin wherein the overall yield of the process is ≥75%. In one aspect, the processes of the invention generate simvastatin wherein the overall chemical cost contribution is about ≤ $150/kg. In one aspect, the processes of the invention generate simvastatin wherein the initial enzymatic hydrolysis of lovastatin runs at about 20% w/v.

In one aspect, the invention provides a process to generate simvastatin comprising the following scheme, or, variations thereof:

Scheme 1. Heterodiacylation Route to Simvastatin



In alternative aspects of the above-illustrated scheme, step 1 can comprise a chemical or an enzymatic hydrolysis; step 2 can comprise a chemical or an enzymatic hydrolysis; step 3 can comprise a chemical or an enzymatic hydrolysis, or, any combination thereof.

In alternative aspects of any of the methods of the invention, at least one step is performed in a reaction vessel. In alternative aspects of any of the methods of the invention, at least one step is performed in a cell extract. In alternative aspects of any of the methods of the invention, at least one step is performed in a whole cell. The cell can be of any source, e.g., a plant cell, a bacterial cell, a fungal cell, a mammalian cell or a yeast cell.

In one aspect of any of the methods of the invention, an ammonium salt of simvastatin is formed.

In one aspect, the methods further comprise re-crystallization of the simvastatin. In one aspect, the methods comprise relactonization to provide simvastatin with a desired purity.

In one aspect of any of the methods of the invention, at least one enzymatic reaction is carried out by a hydrolase (e.g., an esterase or a lipase), for example, a lipase encoded by a nucleic acid having at least 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more, or complete (100%) sequence identity to SEQ ID NO:1, or enzymatically active fragments thereof. In one aspect of any of the methods of the invention, at least one enzymatic reaction is carried out by a hydrolase encoded by a nucleic acid having at least 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more, or complete (100%) sequence identity to SEQ ID NO:3, or enzymatically active fragments thereof.. In one aspect of any of the methods of the invention, at least one enzymatic reaction is carried out by a hydrolase encoded by a nucleic acid having at least 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more, or complete (100%) sequence identity to SEQ ID NO:5, or enzymatically active fragments thereof..

In one aspect of any of the methods of the invention, at least one enzymatic reaction is carried out by a hydrolase(e.g., an esterase or a lipase), for example, a hydrolase having a sequence at least about 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more, or complete (100%) sequence identity to SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6, or enzymatically active fragments thereof.

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims.

All publications, patents, patent applications, GenBank sequences and ATCC deposits, cited herein are hereby expressly incorporated by reference for all purposes.

DESCRIPTION OF DRAWINGS

The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

Figure 1 is an illustration of exemplary protocols for triflate and BF_3 etherate-catalyzed acylation of 4-acetyllactone, as discussed in detail in Example 5, below.

Figure 2 is an illustration the results of several $BF_3.OEt_2$ catalyzed acylations, as Table 3, as discussed in detail in Example 5, below.

Figure 3 is an illustration of Table 4, showing the impurity profile for the product of a 12 g acylation reaction, before and after precipitation, as discussed in detail in Example 5, below.

Figure 4 is an illustration of Table 8, showing data for the isolation of simvastatin, as discussed in detail in Example 5, below.

DETAILED DESCRIPTION

The present invention provides novel synthetic chemical and biochemical processes for the production of Simvastatin and its intermediates. These methods can be efficient and cost-effective.

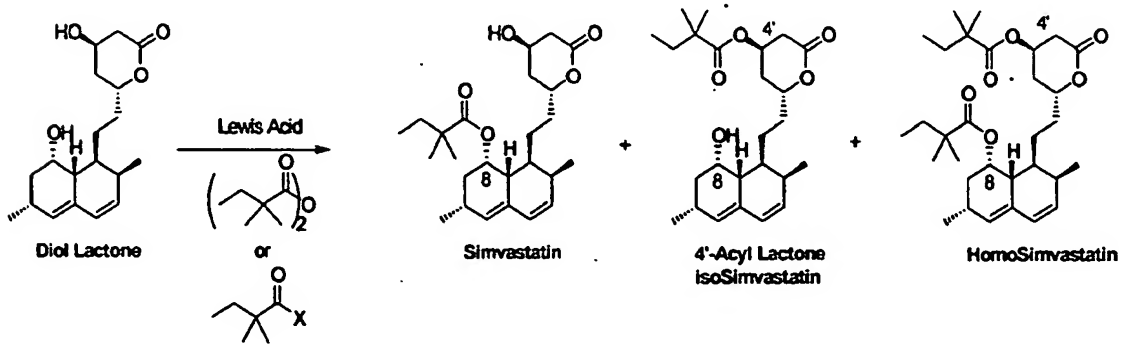
In various aspects of the invention, the methods catalyze reactions biocatalytically using various enzymes, including hydrolases, e.g., acylases and esterases. In one aspect, the invention provides methods for the enzymatic hydrolysis of lovastatin to lovastatin acid using hydrolases. In one aspect, the invention provides methods for the enzymatic acylation of diol lactone to an acyl lactone using hydrolases. In one aspect, the invention provides methods for the enzymatic acylation of an acyl lactone to an acyl simvastatin using hydrolases. In one aspect, the invention provides methods for hydrolyzing a lactone ring using hydrolases.

The invention includes methods for producing simvastatin and various intermediates via *in vitro* or *in vivo* techniques, e.g., whole cells protocols, such as fermentation or other biocatalytic processes.

In one aspect, the invention provides processes comprising a short, convenient route for the conversion of lovastatin into simvastatin, including:

In one aspect, diol lactone made from lovastatin via hydrolysis is regioselectively acylated at the 8-position using a derivative of dimethylbutyric acid and a Lewis acid catalyst. Diol lactone can be made from lovastatin using chemoenzymatic processes described herein.

In one aspect, the invention provides a process comprising:

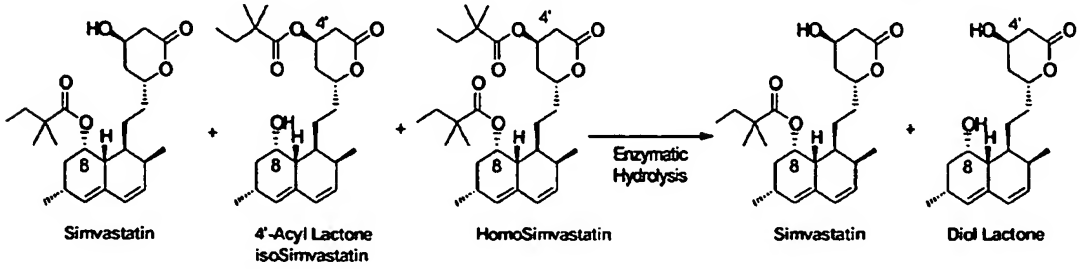


The inventors have found that the treatment of diol lactone with a carboxylic acid derivative in the presence of a Lewis acid catalyst results in predominant acylation at the 8-position. When excess vinyl acetate is used in the presence of a metal triflate, the 8-acetyl derivate is formed almost exclusively at low conversion. Results to date show that the treatment of diol lactone with a combination of dimethylbutyric anhydride, and $Bi(OTf)_3$ or $Cu(OTf)_2$ in dichloromethane at room temperature results in a rapid reaction in which the simvastatin: 4'-acyl lactone ratio is >4:1.

In one aspect, the isolation and purification of simvastatin is by crystallization. In one aspect, the invention provides methods for screening Lewis acid catalysts and/or acylation agents to provide alternative reaction conditions to maximize the

yield of simvastatin and minimize the side products. Maximizing the yield of simvastatin and minimizing the side products helps in crystallization protocols. Use of crystallization to isolate/ purify simvastatin results in an exemplary 2-step process from lovastatin to simvastatin.

In one aspect, the invention provides a process comprising:

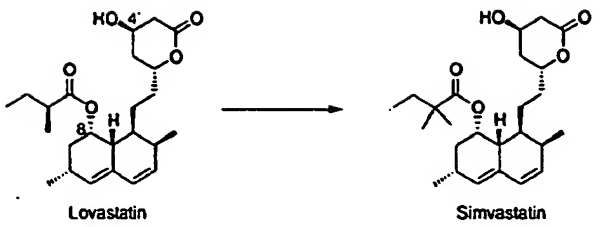


In one aspect, if isosimvastatin and homosimvastatin cannot be reduced to levels that can be purged by crystallization, a final enzymatic hydrolysis step is employed to facilitate the recovery of product. In one aspect, the treatment of mixtures of simvastatin, isosimvastatin and homosimvastatin with an esterase (e.g., enzyme having a sequence as set forth in SEQ ID NO:4, encoded by SEQ ID NO:3), results in the regioselective hydrolysis of the acyl group at the 4'-position, resulting in a mixture of simvastatin and diol lactone. In one aspect, the simvastatin is separated by crystallization.

Alternatively, the use of excess anhydride can be used to push the reaction towards the formation of simvastatin and homosimvastatin. This can minimize the amount of isosimvastatin. Enzymatic hydrolysis of such mixtures results in the formation and ready isolation of simvastatin.

In one aspect of the preparation of simvastatin by regioselective acylation of diol lactone in the presence of Lewis acids, Diol lactone was treated with dimethylbutyric anhydride (0.5 eq) in dichloromethane at room temperature (RT) in the presence of 5 mol% $Cu(OTf)_2$ as catalyst. HPLC analysis indicated 50% conversion of diol lactone within 10 minutes. The ratio of simvastatin (acylation at the 8-position) to isosimvastatin (acylation at the 4-position), was 4:1, with ~4% homosimvastatin being formed.

In one aspect, the invention provides a process comprising:



and alternative aspects, at least one, several or all, of the following steps:

Step 1: Enzymatic hydrolysis of lovastatin, lovastatin acid or a salt of lovastatin acid to form the triol acid using a hydrolase enzyme, e.g., an enzyme of the invention, e.g., SEQ ID NO:4, encoded by SEQ ID NO:3.

Step 2: Heating the triol acid or stirring in the presence of acid to form the diol lactone

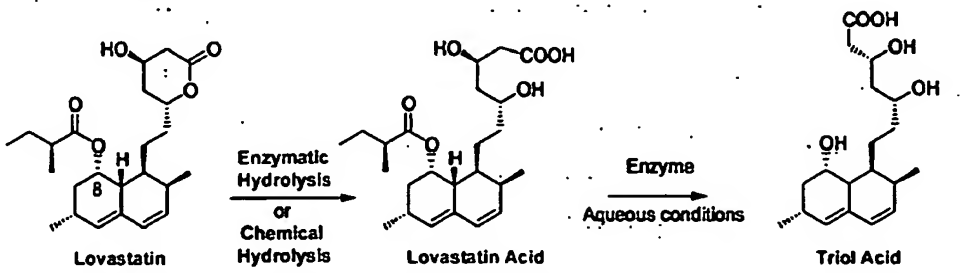
Step 3: Protection of the 4'-OH on the lactone ring by regioselective acylation, either chemically or by using a proprietary or commercially available hydrolase

Step 4: Acylation of the hydroxyl at the 8-position; can be carried out chemically, or enzymatically using a proprietary hydrolase

Step 5: Selective removal of the acyl protecting group at the 4' position, either chemically or enzymatically, yields simvastatin. If necessary, formation of the ammonium salt of simvastatin, and recrystallization of simvastatin, followed by re-lactonization, provides simvastatin with the desired purity.

In one aspect, referring to step 1, as described above, the invention provides a process comprising:

Step 1:



Complete, or substantially complete (in alternative aspects, >99%, >98%, >97% or >96%) removal of the methylbutyrate sidechain may be essential for a process because of the difficulty in separating lovastatin and simvastatin, and the low allowable

levels of lovastatin in simvastatin API. Reported procedures for the hydrolysis of lovastatin require the use of high temperatures and long reaction times for complete reaction.

In one aspect, Lovastatin is hydrolyzed under mild conditions using a hydrolase enzyme (e.g., enzyme having a sequence as set forth in SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6, encoded by SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:5, respectively). This results in hydrolysis of the lactone ring and complete removal of the side-chain in the 8-position. The enzymes having a sequence as set forth in SEQ ID NO:1, SEQ ID NO:3 and SEQ ID NO:5 have been demonstrated to be particularly effective for the enzymatic hydrolysis of the methylbutyrate sidechain: SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6. The enzyme having a sequence as set forth in SEQ ID NO:4 has been subcloned and expressed in different hosts such as *E. coli* and *Pseudomonas*.

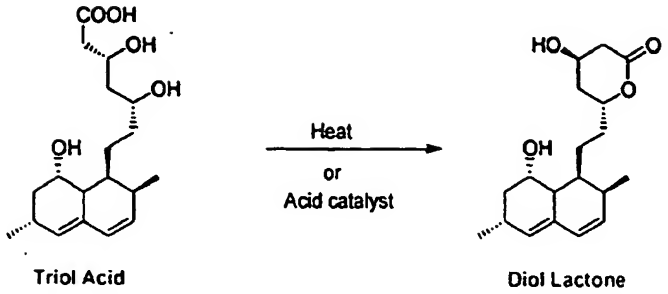
Lovastatin can show poor solubility under the aqueous conditions necessary for enzymatic activity. Thus, in one alternative aspect, a suspension of lovastatin in water is raised to pH >12 to effect a rapid hydrolysis of the lactone ring. This results in the *in-situ* formation of the more soluble lovastatin acid salt. In one aspect, the pH of the reaction mixture is then readjusted downward to a range suitable for the enzymatic reaction; and the enzyme is added.

The enzymatic hydrolysis conditions may also be applied to mixtures of lovastatin and lovastatin acid extracted directly from fermentation broth. Alternatively, the enzyme may be added to the fermentation broth and the triol acid isolated directly.

In one aspect, after hydrolysis, the reaction mixture is carefully acidified. The triol acid can be isolated by extraction and/or filtration and used directly in the next step. Alternatively, it the triol acid is isolated as a solid after a suitable crystallization/precipitation step.

In one aspect, referring to step 2, as described above, the invention provides a process comprising:

Step 2:

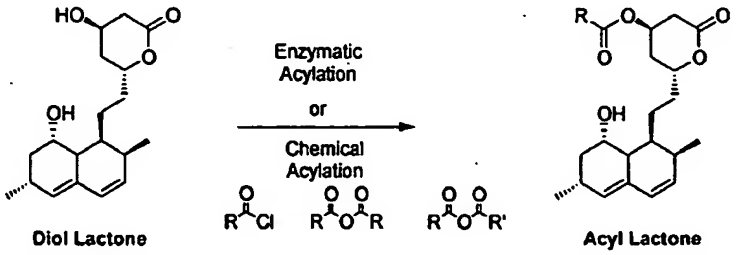


Triol Acid Diol Lactone

In one aspect, the triol acid is re-lactonized by heating in a suitable solvent and driving the equilibrium to the lactone form by removal of water by conventional means. Alternatively, stirring in the presence of a suitable acid will effect closure of the lactone ring.

In one aspect, referring to step 3, as described above, the invention provides a process comprising:

Step 3:



Diol Lactone Acyl Lactone

Regioselective acylation of the hydroxyl group in the 4'-position may be carried out chemically using a carboxylic acid derivative (e.g., acid chloride, symmetric or unsymmetric anhydride etc.), or enzymatically using an enzyme with the desired activity and selectivity, e.g., a hydrolase, such as an esterase. In one aspect, hydrolases (e.g., esterases) are used to acylate diol lactones. The nature of the acyl group can be varied to impart suitable properties, e.g., acetate for ease of removal, benzoate for enhanced crystallinity, formate for enhanced water solubility.

In alternative aspects of the exemplified methods described herein, including the reactions and reagents as illustrated in Steps 3 (supra), 4 and 5 (infra), "R" can be:

(i) - H, a formyl derivative;

(ii) a C1-n alkyl, both straight chain and branched;

(iii) substituted alkyl groups, e.g., chloroacetyl, trichloroacetyl, trifluoroacetyl, methoxyacetyl, phenylacetyl, 4-oxopentyl (levulinate);

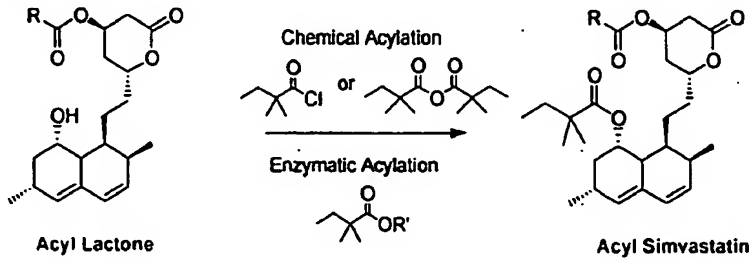
(iv) phenyl and substituted phenyl: e.g., phenyl, p-nitrophenyl;

(v) an R'O- group, forming a carbonate protecting group, exemplified but not limited to: tBuOCO, PhOCO, $PhCH_2OCO$.

In one aspect, an enzyme with enhanced reactivity on long-chain alkyl esters is used when R is a long-chain alkyl group. Solubility may a problem when R is a long-chain alkyl group. In one aspect, R is an acetate, which can be advantageous due to (i) ease of installation, (ii) good enzyme activity for hydrolysis, (iii) solubility, (iv) cost of reagents.

In one aspect, referring to step 4, as described above, the invention provides a process comprising:

Step 4:



In one aspect, a combination of a dimethylbutyric acid derivative with a suitable acylation catalyst (by chemical acylation or enzymatic acylation) is used to install the desired simvastatin side-chain. The combination of dimethylbutyric anhydride/Lewis acid (e.g., $Bi(triflate)_3$, $Cu(triflate)_2$), results in rapid reaction at room temperature (RT).

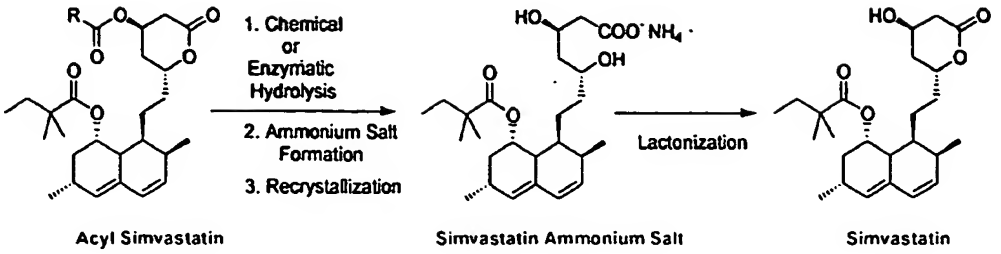
In one aspect, the invention provides methods for screening suitable Lewis acids and reaction conditions, including temperature, solvents etc. Optimum conditions for this acylation for alternative protocols or reagents can be determined using routing screening methods.

In one aspect, enzyme catalyzed acylation of the acyl lactone is used to install the dimethylbutyrate group at the 8-position under very mild conditions (for example, in one aspect, at RT, e.g., about 40°C, using organic solvent), without formation of side products.

The invention provides methods for screening for alternative enzymes that have the desired activity in the methods of the invention. Enzymes can be screened for their effectiveness in various protocols of the invention using routine methods.

In one aspect, referring to step 5, as described above, the invention provides a process comprising:

Step 5:



In one aspect, the final steps require the selective removal of the acyl group at the 4'-position. The acyl group at the 4'-position can be highly susceptible to base-catalyzed elimination, even under only slightly basic conditions. Consequently, the enzymatic hydrolysis has been the most convenient method for regioselective removal of this acyl group. It has been demonstrated that the same enzyme that hydrolyzes lovastatin (SEQ ID NO:4 (encoded by SEQ ID NO:3), in step 1, above) is also an effective catalyst for the selective hydrolysis of acyl groups at the lactone 4'-position. When carried out at pH 7, this enzymatic hydrolysis yields simvastatin with the lactone ring substantially intact.

General Methods

The present invention provides novel biochemical processes for the production of simvastatin and various intermediates. The skilled artisan will recognize that the starting and intermediate compounds used in the methods of the invention can be synthesized using a variety of procedures and methodologies, which are well described in the scientific and patent literature., *e.g.*, Organic Syntheses Collective Volumes, Gilman *et al.* (Eds) John Wiley & Sons, Inc., NY; Venuti (1989) *Pharm Res.* 6:867-873. The invention can be practiced in conjunction with any method or protocol known in the art, which are well described in the scientific and patent literature.

The discussion of the general methods given herein is intended for illustrative purposes only. Other alternative methods and embodiments will be apparent to those of skill in the art upon review of this disclosure.

Enzymes

In one aspect of any of the methods of the invention, at least one enzymatic reaction is carried out by a hydrolase (e.g., an esterase or a lipase), for example, a hydrolase having a sequence at least about 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%,

60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more, or complete (100%) sequence identity to SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6, or enzymatically active fragments thereof.

Enzymes used in the methods of the invention can be produced by any synthetic or recombinant method, or, they may be isolated from a natural source, or, a combination thereof. Nucleic acids encoding enzymes used to practice the methods of the invention, whether RNA, cDNA, genomic DNA, vectors, viruses or hybrids thereof, may be isolated from a variety of sources, genetically engineered, amplified, and/or expressed/ generated recombinantly. Recombinant polypeptides generated from these nucleic acids can be individually isolated or cloned and tested for a desired activity. Any recombinant expression system can be used, including bacterial, mammalian, yeast, insect or plant cell expression systems. Nucleic acids used to practice the methods of the invention can be generated using amplification methods, which are also well known in the art, and include, e.g., polymerase chain reaction, PCR (see, e.g., PCR PROTOCOLS, A GUIDE TO METHODS AND APPLICATIONS, ed. Innis, Academic Press, N.Y. (1990) and PCR STRATEGIES (1995), ed. Innis, Academic Press, Inc., N.Y., ligase chain reaction (LCR) (see, e.g., Wu (1989) Genomics 4:560; Landegren (1988) Science 241:1077; Barringer (1990) Gene 89:117); transcription amplification (see, e.g., Kwoh (1989) Proc. Natl. Acad. Sci. USA 86:1173); and, self-sustained sequence replication (see, e.g., Guatelli (1990) Proc. Natl. Acad. Sci. USA 87:1874); Q Beta replicase amplification (see, e.g., Smith (1997) J. Clin. Microbiol. 35:1477-1491), automated Q-beta replicase amplification assay (see, e.g., Burg (1996) Mol. Cell. Probes 10:257-271) and other RNA polymerase mediated techniques (e.g., NASBA, Cangene, Mississauga, Ontario).

Alternatively, these nucleic acids can be synthesized *in vitro* by well-known chemical synthesis techniques, as described in, e.g., Adams (1983) J. Am. Chem. Soc. 105:661; Belousov (1997) Nucleic Acids Res. 25:3440 3444; Frenkel (1995) Free Radic. Biol. Med. 19:373 380; Blommers (1994) Biochemistry 33:7886 7896; Narang (1979) Meth. Enzymol. 68:90; Brown (1979) Meth. Enzymol. 68:109; Beaucage (1981) Tetra. Lett. 22:1859; U.S. Patent No. 4,458,066.

Techniques for the manipulation of nucleic acids, such as, e.g., subcloning, labeling probes (e.g., random-primer labeling using Klenow polymerase, nick translation, amplification), sequencing, hybridization and the like are well described in the scientific and patent literature, see, e.g., Sambrook, ed., MOLECULAR CLONING: A LABORATORY MANUAL (2ND ED.), Vols. 1-3, Cold Spring Harbor Laboratory, (1989); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Ausubel, ed. John Wiley & Sons, Inc., New York (1997); LABORATORY TECHNIQUES IN BIOCHEMISTRY AND MOLECULAR BIOLOGY: HYBRIDIZATION WITH NUCLEIC ACID PROBES, Part I. Theory and Nucleic Acid Preparation, Tijssen, ed. Elsevier, N.Y. (1993). Another useful means of obtaining and manipulating nucleic acids used to practice the methods of the invention is to clone from genomic samples, and, if desired, screen and re-clone inserts isolated or amplified from, e.g., genomic clones or cDNA clones. Sources of nucleic acid used in the methods of the invention include genomic or cDNA libraries contained in, e.g., mammalian artificial chromosomes (MACs), see, e.g., U.S. Patent Nos. 5,721,118; 6,025,155; human artificial chromosomes, see, e.g., Rosenfeld (1997) Nat. Genet. 15:333-335; yeast artificial chromosomes (YAC); bacterial artificial chromosomes (BAC); P1 artificial chromosomes, see, e.g., Woon (1998) Genomics 50:306-316; P1-derived vectors (PACs), see, e.g., Kern (1997) Biotechniques 23:120-124; cosmids, recombinant viruses, phages or plasmids.

The nucleic acids and proteins of the invention can be detected, confirmed and quantified by any of a number of means well known to those of skill in the art. General methods for detecting both nucleic acids and corresponding proteins include analytic biochemical methods such as spectrophotometry, radiography, electrophoresis, capillary electrophoresis, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), hyperdiffusion chromatography, and the like, and various immunological methods such as fluid or gel precipitin reactions, immunodiffusion (single or double), immunoelectrophoresis, radioimmunoassays (RIAs), enzyme-linked immunosorbent assays (ELISAs), immunofluorescent assays, and the like. The detection of nucleic acids and polypeptides can be by well known methods such as Southern analysis, northern analysis, gel electrophoresis, PCR, radiolabeling, scintillation counting, and affinity chromatography.

In one step of an exemplary method of the invention, an esterase is used. Any esterase, or enzyme (e.g., a hydrolase) or other polypeptide having a similar activity can be used.

Any method for screening for enzymes for use in the methods of the invention, e.g., enzymes for the hydrolysis of lovastatin, lovastatin acid, 4-acetyl simvastatin or simvastatin, can be used, and, these methods are well known in the art. For example, in one exemplary set of screen conditions used to determine an enzyme(s) to be used in a method of the invention comprises use of 2.5 mM substrate, 100 mM phosphate buffer/ co-solvent pH 7 to pH 8, 30°C, 48 h, with the following composition: (i) lovastatin or simvastatin in MTBE/buffer, (ii) lovastatin or simvastatin in toluene/buffer, (iii) lovastatin acid or simvastatin acid in 10% methanol/buffer. Screen results were confirmed at 1 mM substrate.

Using this exemplary assay, it was determined that three enzymes having sequences as set forth in SEQ ID NO:2, SEQ ID NO:4 and SEQ ID NO:6, were active for the hydrolysis of lovastatin or lovastatin acid. Only an enzyme having a sequence as set forth in SEQ ID NO:4 showed activity for the hydrolysis of simvastatin. SEQ ID NO:4 and SEQ ID NO:2 were further evaluated at 25, 50 and 100 mM lovastatin acid in 10% MeOH/buffer, pH 9, the more soluble lovastatin acid being used as substrate for convenience. SEQ ID NO:4 showed high conversion of substrate in many cases, with solution yields of 12-60% triol acid. SEQ ID NO:2 showed no reaction under the same conditions. SEQ ID NO:4 also showed some activity (~10% conversion at best) on simvastatin.

Genomic clones comprising sequences encoding enzymes having sequences as set forth in SEQ ID NO:4, SEQ ID NO:2 , SEQ ID NO:6 (e.g., encoded by exemplary SEQ ID NO:3, SEQ ID NO:1, and SEQ ID NO:5, respectively), were compared for the hydrolysis of lovastatin acid under standard conditions (the same total protein concentration, or the same enzyme activity normalized against the fluorescent substrate, methylumbelliferyl butyrate (MUB)). Enzymes having a sequence comprising SEQ ID NO:4 showed the best activity under the reaction conditions.

The genomic clones comprising sequences encoding enzymes having sequences as set forth in SEQ ID NO:4 and SEQ ID NO:2 (e.g., encoded by exemplary SEQ ID NO:3 and SEQ ID NO:1, respectively), were subcloned. SEQ ID NO:2 has a leader

sequence which is believed to be required for secretion/localization, and was subcloned with and without the leader sequence. The subclones were assayed against MUB and lovastatin acid; only the SEQ ID NO:2- encoding subclone with the leader sequence showed activity against MUB. Furthermore, none of the subclones showed activity on lovastatin acid.

Transposon insertion experiments with the genomic clone comprising a nucleic acid encoding SEQ ID NO:4 identified the gene responsible for the lovastatin esterase activity. This gene encoded an esterase with a predicted 43 kD molecular weight; the identity was further confirmed by isolating the 43 kD band from a native gel and confirming activity on lovastatin acid and by MS analysis. The *E. coli* construct comprising a nucleic acid encoding SEQ ID NO:4 was capable of hydrolyzing lovastatin to give a 93-98% conversion to triol acid in 21h at 35°C at 350 mM substrate.

Capillary Arrays

The methods of the invention, and/or, screening protocols used to determine enzyme(s) to be used in a method of the invention, can be practiced in whole or in part by capillary arrays, such as the GIGAMATRIX™, Diversa Corporation, San Diego, CA. See, e.g., WO0138583. Reagents or polypeptides (e.g., enzymes) can be immobilized to or applied to an array, including capillary arrays. Capillary arrays provide another system for holding and screening reagents, catalysts (e.g., enzymes) and products. The apparatus can further include interstitial material disposed between adjacent capillaries in the array, and one or more reference indicia formed within of the interstitial material. High throughput screening apparatus can also be adapted and used to practice the methods of the invention, see, e.g., U.S. Patent Application No. 20020001809.

Whole Cell-Based Methods

The methods of the invention can be practiced in whole or in part in a whole cell environment. The invention also provides for whole cell evolution, or whole cell engineering, of a cell to develop a new cell strain having a new phenotype to be used in the methods of the invention, e.g., a new cell line comprising one, several or all enzymes used in a method of the invention. This can be done by modifying the genetic composition of the cell, where the genetic composition is modified by addition to the cell of a nucleic acid, e.g., a coding sequence for an enzyme used in the methods of the invention. See, e.g., WO0229032; WO0196551.

The host cell for the "whole-cell process" may be any cell known to one skilled in the art, including prokaryotic cells, eukaryotic cells, such as bacterial cells, fungal cells, yeast cells, mammalian cells, insect cells, or plant cells.

To detect the production of an intermediate or product of the methods of the invention, or a new phenotype, at least one metabolic parameter of a cell (or a genetically modified cell) is monitored in the cell in a "real time" or "on-line" time frame by Metabolic Flux Analysis (MFA). In one aspect, a plurality of cells, such as a cell culture, is monitored in "real time" or "on-line." In one aspect, a plurality of metabolic parameters is monitored in "real time" or "on-line."

Metabolic flux analysis (MFA) is based on a known biochemistry framework. A linearly independent metabolic matrix is constructed based on the law of mass conservation and on the pseudo-steady state hypothesis (PSSH) on the intracellular metabolites. In practicing the methods of the invention, metabolic networks are established, including the:

- identity of all pathway substrates, products and intermediary metabolites
- identity of all the chemical reactions interconverting the pathway metabolites, the stoichiometry of the pathway reactions,
- identity of all the enzymes catalyzing the reactions, the enzyme reaction kinetics,
- the regulatory interactions between pathway components, e.g. allosteric interactions, enzyme-enzyme interactions etc,
- intracellular compartmentalization of enzymes or any other supramolecular organization of the enzymes, and,
- the presence of any concentration gradients of metabolites, enzymes or effector molecules or diffusion barriers to their movement.

Once the metabolic network for a given strain is built, mathematic presentation by matrix notion can be introduced to estimate the intracellular metabolic fluxes if the on-line metabolome data is available. Metabolic phenotype relies on the changes of the whole metabolic network within a cell. Metabolic phenotype relies on the change of pathway utilization with respect to environmental conditions, genetic regulation, developmental state and the genotype, etc. In one aspect of the methods of the invention,

after the on-line MFA calculation, the dynamic behavior of the cells, their phenotype and other properties are analyzed by investigating the pathway utilization.

Control of physiological state of cell cultures will become possible after the pathway analysis. The methods of the invention can help determine how to manipulate the fermentation by determining how to change the substrate supply, temperature, use of inducers, etc. to control the physiological state of cells to move along desirable direction. In practicing the methods of the invention, the MFA results can also be compared with transcriptome and proteome data to design experiments and protocols for metabolic engineering or gene shuffling, etc. Any aspect of metabolism or growth can be monitored.

Monitoring expression of an mRNA transcript

In one aspect of the invention, the engineered phenotype comprises increasing or decreasing the expression of an mRNA transcript or generating new transcripts in a cell. This increased or decreased expression can be traced by use of a fluorescent polypeptide, e.g., a chimeric protein comprising an enzyme used in the methods of the invention. mRNA transcripts, or messages, also can be detected and quantified by any method known in the art, including, e.g., Northern blots, quantitative amplification reactions, hybridization to arrays, and the like. Quantitative amplification reactions include, e.g., quantitative PCR, including, e.g., quantitative reverse transcription polymerase chain reaction, or RT-PCR; quantitative real time RT-PCR, or "real-time kinetic RT-PCR" (see, e.g., Kreuzer (2001) Br. J. Haematol. 114:313-318; Xia (2001) Transplantation 72:907-914).

In one aspect of the invention, the engineered phenotype is generated by knocking out expression of a homologous gene. The gene's coding sequence or one or more transcriptional control elements can be knocked out, e.g., promoters enhancers. Thus, the expression of a transcript can be completely ablated or only decreased.

In one aspect of the invention, the engineered phenotype comprises increasing the expression of a homologous gene. This can be effected by knocking out of a negative control element, including a transcriptional regulatory element acting in cis- or trans- , or, mutagenizing a positive control element. One or more, or, all the transcripts of a cell can be measured by hybridization of a sample comprising transcripts of the cell, or, nucleic acids representative of or complementary to transcripts of a cell, by hybridization to immobilized nucleic acids on an array.

Monitoring expression of a polypeptides, peptides and amino acids

In one aspect of the invention, the engineered phenotype comprises increasing or decreasing the expression of a polypeptide or generating new polypeptides in a cell. This increased or decreased expression can be traced by use of a fluorescent polypeptide, e.g., a chimeric protein comprising an enzyme used in the methods of the invention. Polypeptides, reagents and end products (e.g., simvastatin) also can be detected and quantified by any method known in the art, including, e.g., nuclear magnetic resonance (NMR), spectrophotometry, radiography (protein radiolabeling), electrophoresis, capillary electrophoresis, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), hyperdiffusion chromatography, various immunological methods, e.g. immunoprecipitation, immunodiffusion, immuno-electrophoresis, radioimmunoassays (RIAs), enzyme-linked immunosorbent assays (ELISAs), immuno-fluorescent assays, gel electrophoresis (e.g., SDS-PAGE), staining with antibodies, fluorescent activated cell sorter (FACS), pyrolysis mass spectrometry, Fourier-Transform Infrared Spectrometry, Raman spectrometry, GC-MS, and LC-Electrospray and cap-LC-tandem-electrospray mass spectrometries, and the like. Novel bioactivities can also be screened using methods, or variations thereof, described in U.S. Patent No. 6,057,103. Polypeptides of a cell can be measured using a protein array.

Determining the degree of sequence identity

In one aspect of any of the methods of the invention, at least one enzymatic reaction is carried out by a hydrolase (e.g., an esterase, or acylase) encoded by a nucleic acid having at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more, or complete (100%) sequence identity to SEQ ID NO:1, SEQ ID NO:3 and/or SEQ ID NO:5, or enzymatically active fragments thereof. In one aspect of any of the methods of the invention, at least one enzymatic reaction is carried out by a hydrolase (e.g., an esterase, or acylase) having a sequence at least about 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%,

or more, or complete (100%) sequence identity to SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6, or enzymatically active fragments thereof..

Enzymatic activity can be determined by routine screening using known protocols, or, the methods of the invention, as described herein. For example, enzymatic activity can be determined by testing whether a polypeptide or peptide can hydrolyze a lactone ring, or, enzymatically acylate a diol lactone, as described herein.

Protein and/or nucleic acid sequence homologies may be evaluated using any of the variety of sequence comparison algorithms and programs known in the art. Such algorithms and programs include, but are by no means limited to, TBLASTN, BLASTP, FASTA, TFASTA, and CLUSTALW (see, e.g., Pearson (1988) Proc. Natl. Acad. Sci. USA 85(8):2444-2448; Altschul (1990) J. Mol. Biol. 215(3):403-410; Thompson (1994) Nucleic Acids Res. 22(2):4673-4680; Higgins *et al.*, Methods Enzymol. 266:383-402, 1996; Altschul *et al.*, J. Mol. Biol. 215(3):403-410, 1990; Altschul *et al.*, Nature Genetics 3:266-272, 1993).

Homology or identity is often measured using sequence analysis software (*e.g.*, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705). Such software matches similar sequences by assigning degrees of homology to various deletions, substitutions and other modifications. The terms "homology" and "identity" in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same when compared and aligned for maximum correspondence over a comparison window or designated region as measured using any number of sequence comparison algorithms or by manual alignment and visual inspection.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

A "comparison window", as used herein, includes reference to a segment of any one of the numbers of contiguous residues. For example, in alternative aspects of the invention, contiguous residues ranging anywhere from about 20 to the full length of an exemplary polypeptide or nucleic acid sequence of the invention are compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. If the reference sequence has the requisite sequence identity to an exemplary polypeptide or nucleic acid sequence of the invention, e.g., 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5 or SEQ ID NO:6, and the sequence is or encodes a hydrolase, that sequence can be used in at least one step of a method of the invention. In alternative embodiments, subsequences ranging from about 20 to 600, about 50 to 200, and about 100 to 150 are compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequence for comparison are well known in the art. Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith & Waterman, Adv. Appl. Math. 2:482, 1981, by the homology alignment algorithm of Needleman & Wunsch, J. Mol. Biol. 48:443, 1970, by the search for similarity method of person & Lipman, Proc. Nat'l. Acad. Sci. USA 85:2444, 1988, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection. Other algorithms for determining homology or identity include, for example, in addition to a BLAST program (Basic Local Alignment Search Tool at the National Center for Biological Information), ALIGN, AMAS (Analysis of Multiply Aligned Sequences), AMPS (Protein Multiple Sequence Alignment), ASSET (Aligned Segment Statistical Evaluation Tool), BANDS, BESTSCOR, BIOSCAN (Biological Sequence Comparative Analysis Node), BLIMPS (BLocks IMProved Searcher), FASTA, Intervals & Points, BMB, CLUSTAL V, CLUSTAL W, CONSENSUS, LCONSENSUS, WCONSENSUS, Smith-Waterman algorithm, DARWIN, Las Vegas algorithm, FNAT (Forced Nucleotide Alignment Tool), Framealign,

Framesearch, DYNAMIC, FILTER, FSAP (Fristensky Sequence Analysis Package), GAP (Global Alignment Program), GENAL, GIBBS, GenQuest, ISSC (Sensitive Sequence Comparison), LALIGN (Local Sequence Alignment), LCP (Local Content Program), MACAW (Multiple Alignment Construction & Analysis Workbench), MAP (Multiple Alignment Program), MBLKP, MBLKN, PIMA (Pattern-Induced Multi-sequence Alignment), SAGA (Sequence Alignment by Genetic Algorithm) and WHAT-IF. Such alignment programs can also be used to screen genome databases to identify polynucleotide sequences having substantially identical sequences. Databases containing genomic information annotated with some functional information are maintained by different organization, and are accessible via the internet.

BLAST, BLAST 2.0 and BLAST 2.2.2 algorithms are also used to practice the invention. They are described, e.g., in Altschul (1977) Nuc. Acids Res. 25:3389-3402; Altschul (1990) J. Mol. Biol. 215:403-410. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul (1990) supra). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always >0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, M=5, N=-4 and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength of 3, and expectations (E) of 10, and the BLOSUM62 scoring matrix

(see Henikoff & Henikoff (1989) Proc. Natl. Acad. Sci. USA 89:10915) alignments (B) of 50, expectation (E) of 10, M=5, N= -4, and a comparison of both strands. The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, e.g., Karlin & Altschul (1993) Proc. Natl. Acad. Sci. USA 90:5873). One measure of similarity provided by BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a references sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001. In one aspect, protein and nucleic acid sequence homologies are evaluated using the Basic Local Alignment Search Tool ("BLAST"). For example, five specific BLAST programs can be used to perform the following task: (1) BLASTP and BLAST3 compare an amino acid query sequence against a protein sequence database; (2) BLASTN compares a nucleotide query sequence against a nucleotide sequence database; (3) BLASTX compares the six-frame conceptual translation products of a query nucleotide sequence (both strands) against a protein sequence database; (4) TBLASTN compares a query protein sequence against a nucleotide sequence database translated in all six reading frames (both strands); and, (5) TBLASTX compares the six-frame translations of a nucleotide query sequence against the six-frame translations of a nucleotide sequence database. The BLAST programs identify homologous sequences by identifying similar segments, which are referred to herein as "high-scoring segment pairs," between a query amino or nucleic acid sequence and a test sequence which is preferably obtained from a protein or nucleic acid sequence database. High-scoring segment pairs are preferably identified (i.e., aligned) by means of a scoring matrix, many of which are known in the art. Preferably, the scoring matrix used is the BLOSUM62 matrix (Gonnet et al., Science 256:1443-1445, 1992; Henikoff and Henikoff, Proteins 17:49-61, 1993). Less preferably, the PAM or PAM250 matrices may also be used (see, e.g., Schwartz and Dayhoff, eds., 1978, Matrices for Detecting Distance Relationships: Atlas of Protein Sequence and Structure, Washington: National Biomedical Research Foundation).

In one aspect of the invention, the NCBI BLAST 2.2.2 programs is used, default options to blastp. There are about 38 setting options in the BLAST 2.2.2 program. In

this exemplary aspect of the invention, all default values are used except for the default filtering setting (i.e., all parameters set to default except filtering which is set to OFF); in its place a "-F F" setting is used, which disables filtering. Use of default filtering often results in Karlin-Altschul violations due to short length of sequence.

The default values used in this exemplary aspect of the invention include:

"Filter for low complexity: ON
Word Size: 3
Matrix: Blosum62
Gap Costs: Existence:11
Extension:1"

Other default settings can be: filter for low complexity OFF, word size of 3 for protein, BLOSUM62 matrix, gap existence penalty of -11 and a gap extension penalty of -1. An exemplary NCBI BLAST 2.2.2 program setting has the "-W" option default to 0. This means that, if not set, the word size defaults to 3 for proteins and 11 for nucleotides.

The invention will be further described with reference to the following examples; however, it is to be understood that the invention is not limited to such examples.

EXAMPLES

Example 1: Chemoenzymatic production of Simvastatin

The following example describes an exemplary protocol of the invention for the chemoenzymatic production of Simvastatin.

Enzymatic Hydrolysis of Lovastatin (Step 1, above)

The enzyme having a sequence as set forth in SEQ ID NO:4 (encoded by SEQ ID NO:3) was evaluated at 0.1 to 0.5 M concentrations of lovastatin or lovastatin acid in 7-10% MeOH/buffer, with the reaction being maintained at pH 9-9.5 by automatic addition of base. The best result was obtained at 0.5M lovastatin on a 500 mL scale using a lyophilized preparation of enzyme SEQ ID NO:4 (centrifuged supernatant from lysed cells) containing 14 mg/mL total protein; complete conversion of substrate was observed after 48 h.

The reaction mixture was acidified (pH 2), and the precipitate collected by centrifugation and dried. The filtrate was extracted with iPrOAc and the organic extract was added to the dried filter cake. The resulting suspension was heat to reflux in a Dean-Stark

apparatus until lactonization was complete. The resulting solution was filtered through a Celite pad, and the filtrate was washed with satd. $NaHCO_3$. The resulting iPrOAc solution was concentrated until (x 0.5), diluted with hexanes and cooled to 0°C. The precipitated solid was filtered and air-dried to yield diol lactone (63 g, 79.5% isolated yield; another 10.3 g of product was identified in various washes and mother liquors). The product contained <1% lovastatin.

Enzymatic Acylation of Diol Lactone (Step 3, above)

A mixture of diol lactone (25 mM), vinyl acetate (250 mM) and *Candida antarctica* lipase B (33 mg) in TBME (1 mL) was shaken at RT. After 44 h HPLC indicated the formation of the monoacetate with 60% conversion.

Acetylation of Diol Lactone (Step 3, above)

Diol lactone (10 g, 31.25 mmol,) and DMAP (0.5727 g, 4.69 mmol, 15 mol%) were dissolved in anhydrous CH_2Cl_2 (62.5 ml), stirred under N_2 and cooled to 0°C. The pivalic/acetic mixed anhydride (4.95, 34.4 mmol, 1.1 equivalent) was added in two portions. The first portion (2 ml) was added in one shot, followed by the rest of anhydride added by syringe pump over 20 min. The reaction mixture was stirred 0°C for 30min, at ambient temperature for 1.5 hours. The reaction was quenched by adding 31.2ml water and stirred for 10min at ambient temperature, then the mixture was transferred into a separation funnel and the organic layer was washed sequentially by 5% HCl (31.3 ml), saturated $NaHCO_3$ (32 ml) and brine (32 ml). The organic layer was collected and dried over Na_2SO_4, concentrated after the removal of drying agent by filtration. The residue was dried *in vacuo* overnight. A slightly yellowish solid was obtained (10.8g, yield 95.5%). HPLC analysis indicated the following distribution of products: 4-acetyl lactone (95.3%), diol lactone (2.1%), 4, 8'-diacetyl lactone (1.2%), elimination (1.4%).

Preparation of Acetyl Simvastatin (Step 4, above)

4-Acetyl lactone was dried under vacuum overnight at room temperature, stored under nitrogen, then dissolved in anhydrous methylene chloride (1g/2.5-3ml ratio) at room temperature under nitrogen. Meanwhile, $Cu(OTf)_2$ (5mol%) was dissolved in the minimum amount of acetonitrile at room temperature, then 1.05-1.2 eq of dimethylbutyric anhydride was added to the solution, stirring at room temperature for 30 min to hour. This

$Cu(OTf)_2$/anhydride solution was transferred into the 4-Acetyl lactone solution through syringe at room temperature under nitrogen with stirring. When complete (monitored by HPLC), the reaction was quenched by addition of water, and washed with satd., $NaHCO_3$ The isolated organic layer was dried over Na_2SO_4, filtered and evaporated to obtain crude 4-acetyl simvastatin (>99%).

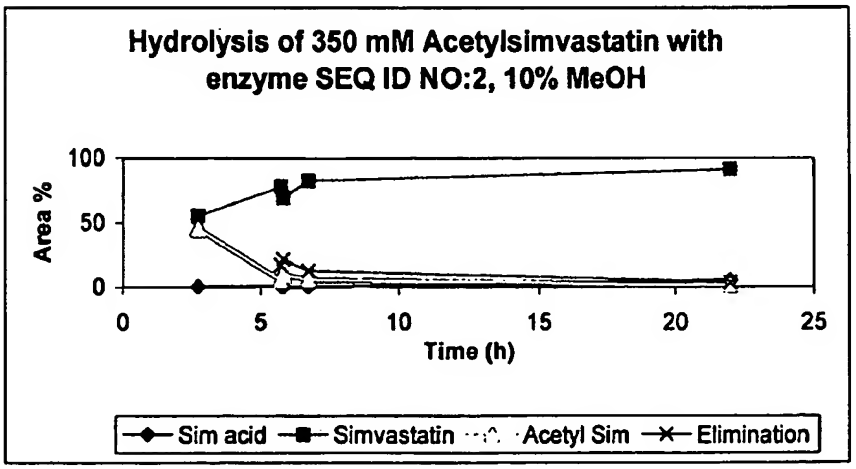
Enzymatic Hydrolysis of Acetyl Simvastatin (Step 5, above)

3.22 g Acetylsimvastatin (final concentration 350 mM)

2 ml MeOH; 100 µl 4M Tris; 9.9 ml water

8 ml BD12785 (125 mg/ml lyophilized lysate in water)

The reaction is performed in a 25 ml vessel with overhead stirring and a magnetic stirrer bar. pH-stat conditions are maintained by a DasGip STIRRER-PRO® system; a pH of 7 is maintained by addition of 10% NH_4OH. As the conversion approaches ~75%, 4 ml of toluene are added to solubilize the material. The reaction is allowed to proceed overnight, at which time further solvent (toluene or methylene chloride) is added to ensure that all insoluble material is dissolved. A sample is analyzed by HPLC.



Final composition of the reaction: Simvastatin acid 4.7%, Simvastatin 90.9%, Acetyl simvastatin 0.9%, Putative elimination product of simvastatin 3.5%. Final conversion 95.6%.

Example 2: Lovastatin Esterase Assay

In one aspect, the invention provides methods comprising the enzymatic hydrolysis of lovastatin, lovastatin acid or a salt of lovastatin acid to form the triol acid using a hydrolase enzyme, e.g., an enzyme of the invention, e.g., SEQ ID NO:4, encoded by SEQ ID NO:3. In one aspect, the invention provides methods comprising the enzymatic hydrolysis of lovastatin, lovastatin acid or a salt of lovastatin acid to form simvastatin. The following example describes an exemplary lovastatin esterase assay which can be used to practice the methods of the invention For example, this exemplary assay can be used to determine is a hydrolase enzyme, e.g., an esterase, can be used to practice a method of the invention.

(a) Cell Lysis (assay scale):

An ice-cold lysis solution (enough for 9 samples) was prepared from B-PER (4.5 µL) (Pierce, #78248), lysozyme (200 µL) (Sigma, L-6876; stock solution 10 mg/ml), and DNAse I (40 µL) (Sigma, DN-25; stock solution 5 mg/mL).

Meanwhile 50 µL of culture was resuspended by vortex in 950 µL water and centrifuged for 15 min at 4°C at 16,000g. The resulting cell pellet was resuspended in 500 µL lysis solution by pipet. The sample was incubated on ice for 45 min before proceeding with activity analysis.

(b) Total Protein Quantitation

The protein quantitation can be done by any Coomassie dye based assay using the Bradford method; the kit used in this instance was the Coomassie Plus Protein Assay Kit (Pierce, #23236). This was used according to the manufacturer's guidelines (available from Pierce , Doc #0229).

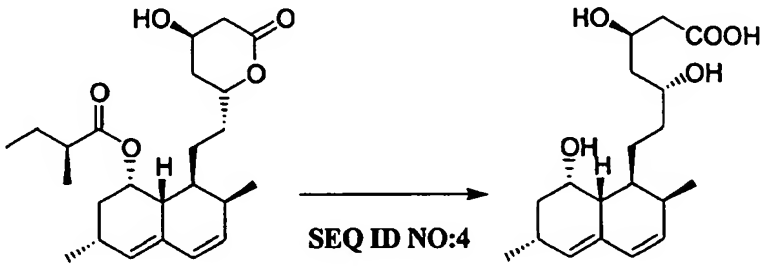
The protein solution of interest was diluted to within the linear range of a standard (albumin) of known protein concentration measured simultaneously. Once the protein concentration was known, an appropriate dilution was calculated to permit reasonable pipetting of 0.1 micrograms of total protein (i.e. within the range of 2 to 20 µL).

(c) Enzyme Activity: Methyl Umbelliferyl butyrate (MUB) Hydrolysis

The volume required for 0.1 µg total protein is brought to 25 µL with 50 mM Tris-HCl pH 9 buffer (buffer type/pH are flexible) in a 96 well plate. Meanwhile a stock of 4mM MUB (9.8 mg in 10mL DMSO) is made and apportioned in 400 µL aliquots to be

stored at -20°C. The stock is diluted to a working concentration of 200 μM: 400 μL in 7.6 mL 10 mM HEPES buffer pH 7.0. To the 25 μL sample is added 25 μL of the working MUB solution immediately before reading kinetically over a 300s period on a fluorescent plate reader (SpectraMAX GeminiXS: λ_{ex} = 360nm; λ_{em} = 465nm). The working solution can be stored at 4°C for several days before degradation occurs. It is preferable to thaw an aliquot of DMSO stock and make fresh working solution before each assay.

Hydrolysis of Lovastatin by SEQ ID NO:4 (100 g Scale) (2787-40):



$C_{24}H_{36}O_5$
Mol. Wt.: 404.54

$C_{19}H_{30}O_5$
Mol. Wt.: 338.44

1. Lovastatin (10x10 g, 0.25 mol) and water (13x10 mL) were slowly added in alternating portions to a rapidly stirring mixture of MeOH (35 mL, 7% final volume) and 6M NaOH (43 mL, 0.26 mol) in a 1L 3-neck flask equipped with an overhead paddle stirrer.
2. When a homogeneous mixture was obtained, the mixture was stirred at 35°C until the pH dropped to 8 (approx. 2h) whereupon lovastatin was converted to lovastatin acid.
3. Meanwhile lyophilized enzyme (22.64 g) was reconstituted with water (final volume 180 mL). 4M Tris (4 mL) and the reconstituted enzyme solution were added to the lovastatin acid solution. Water (108 mL) was added to bring the volume to 500 mL before initiating pH control.
4. The reaction was controlled using a DASGIP AG

5. -PRO® bioreactor using 30% NH_4OH to maintain pH 9.5. The reaction was stirred for 48 h (Note 1, below) and maintained at 35°C, aliquots (10 µL quenched in MeOH, 990 µL) being taken periodically to monitor progress of the reaction by HPLC (Note 2, below).
6. The reaction was terminated by transferring to a 4L beaker and diluting it with water (1L). The pH of the mixture was adjusted with 6M HCl. At pH ~4.4 the mixture became very viscous as a white solid precipitated and stirring rate was increased to prevent "gelling" of the mixture. The mixture was adjusted to pH 2.5 using a total of 120 mL 6M HCl and stirred for a further 0.5 h.
7. The resulting slurry was filtered through Whatman #1 filter paper on a 21 cm Buchner funnel, and the damp filter cake washed with water (0.5 L). The damp filter cake was allowed air dry for ~1 h; it was then transferred into 4 x 600 mL lyophilizer flasks and dried on a lyophilizer for 48 h to provide an off-white powder (98.6 g) (Note 3, below).
8. The filtrate was divided into 3 equal portions which were extracted with a single portion of EtOAc (500 mL). While the 1st extraction separated easily, the 2nd and 3rd portions formed emulsions which did not separate cleanly even after treatment with satd. NaCl (100 mL). The EtOAc extract was washed with saturated ("satd") NaCl (100 mL), dried (Na_2SO_4) and filtered. The filtrate was stirred under N_2 and a solution of $MeSO_3H$ (0.2 mL, 3.1 mmol; final concentration ~7 mM) in EtOAc (5 mL) was added dropwise over a period of ~5 minutes. After 4.5 h the reaction solution was washed with satd. $NaHCO_3$ (200 mL), water (100 mL) and satd. NaCl (100 mL). The EtOAc layer was concentrated to ~50 mL on a rotary evaporator and diol lactone was precipitated by the slow dropwise addition of hexanes (200 mL). The precipitated solid was collected by filtration and dried (3.36 g, 81.3% purity); a further 0.26 g remained in the mother liquors.
9. The total yield was determined to be 94.9% (see Note 4, below).

Notes

1. HPLC indicated that reactions on a 100 g scale were ~97% complete after 22h, but were often allowed stir for longer to ensure complete hydrolysis,
2. Samples were analyzed on a Waters 1100 Series HPLC equipped with a DAD, using a Zorbax SB-Phenyl column (4.6 x 75 mm)(45% MeCN/0.1% H_3PO_4 isocratic; 1 ml/min; 30°C; 238 nm). The order of elution was: Triol acid: 1.4 min, Diol lactone: 1.9 min, Lovastatin Acid: 3.8 min, Lovastatin: 7.3 min.

3. The filter cake at this stage consists of crude triol acid and precipitated protein.
4. The total yield of product was calculated as shown in the Table:

		g	Purity %	mmol	
Starting material	Lovastatin	100	100	247	
Products	Triol Acid	98.6	77.8[#]	225	
	Isolated Diol Lactone	3.36	81.5[•]	8.5	
	Diol lactone in mother liquors	0.26		0.8	
Total				234.3	94.9%

[#] Assayed by ^{1}H NMR versus toluic acid as an internal standard

• Assayed by HPLC versus a working standard

Hydrolysis of Lovastatin by SEQ ID NO:4 (150 g Scale) (2787-60)

1. Lovastatin (150 g, 0.37 mol) and water (300 mL) were slowly added in alternating portions to a rapidly stirring mixture of MeOH (52.5 mL) and 50% w/w NaOH (30 mL, 0.57 mol) in a 1L 3-neck flask equipped with an overhead paddle stirrer. The reaction was stirred at room temperature overnight and the clear mixture then acidified to pH ~7-8 using conc. HCl (~25 mL) (Note 1, below).
2. SEQ ID NO:4 (17 g) was reconstituted in water (50 ml water) and added to the reaction. A further portion of water (300 mL) to bring the volume of the reaction to a total of 750 mL.
3. The reaction was controlled using a DASGIP AG FEDBATCH -pro® bioreactor using 30% NH_4OH to maintain pH 9.5. The reaction was stirred and maintained at 35°C, aliquots (10 µL quenched in MeOH, 990 µL) being taken periodically to monitor progress of the reaction by HPLC (Note 2, below).

and vigorously stirred. The mixture was acidified to pH 2.5 with 6M HCl (160 mL) and stirred at room temperature for a further 1.5 h.

5. The slurry was filtered through Whatman #1 filter paper on a 19 cm Buchner funnel and the damp filter cake washed with water (0.5 L). The mixture filtered easily to give a cream-colored filter cake and a golden yellow filtrate. The damp filter cake was allowed air dry for ~1 h; it was then transferred into 4 x 600 mL lyophilizer flasks and dried on a lyophilizer to provide an off-white powder (154.8 g) (Note 3, below).
6. The filtrate was divided into 3 equal portions which were extracted with a single portion of EtOAc (600 mL). The EtOAc extract was washed with satd. NaCl (100 mL), dried (Na_2SO_4), filtered and concentrated to ~250 mL. The filtrate was stirred under N_2 and a solution of $MeSO_3H$ (0.2 mL, 3.1 mmol; final concentration ~15 mM) in EtOAc (4 mL) was added dropwise over a period of ~5 minutes. After 70 min. the reaction solution was washed with satd. $NaHCO_3$ (200 mL), and satd. NaCl (50 mL). The EtOAc solution was allowed stand overnight, decanted, and concentrated to ~120 mL on a rotary evaporator. The diol lactone was precipitated by the slow dropwise addition of hexanes (200 mL). The precipitated solid was filtered and dried (3.22 g, 92.3% purity); a further 0.47 g remained in the mother liquors.
7. The total yield was determined to be 98.9% (see Note 4, below).

Notes

1. Unlike previous reactions, 50% w/w NaOH solution was inadvertently used to open the lactone ring, resulting in an excess of NaOH and a high pH which had to be lowered before the enzymatic reaction could be initiated at pH9.5.
2. Due to a temporary shortage of enzyme, this reaction was under-charged with only 17 g enzyme rather than the usual 30 g. The reaction proceeded more slowly and more enzyme was added at 43.5 h (5 g) and 64 h (3 g).
3. The filter cake at this stage consists of crude triol acid and precipitated protein.

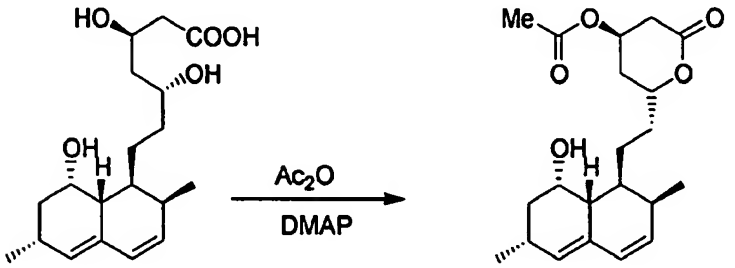
4. The total yield of product was calculated as shown in the Table:

		g	Purity %	mmol	
Starting material	Lovastatin	150	100	371	
Products	Triol Acid	154.8	77.8#	356	
	Isolated Diol Lactone	3.22	92.3*	9.3	
	Diol lactone in mother liquors	0.47		1.5	
Total				366.8	98.9%

Assayed by ^{1}H NMR versus toluic acid as an internal standard

* Assayed by HPLC versus a working standard

Example 3: SYNTHESIS OF 4-ACETYL DIOL LACTONE



$C_{19}H_{30}O_5$
Mol. Wt.: 338.44

$C_{21}H_{30}O_5$
Mol. Wt.: 362.46

A. Direct Acetylation of Triol Acid (20 g scale) (2516-51)

1. Crude triol acid (25.82 g, 59.1 mmol) (Note 1, below) was charged to a dry 500 mL round bottom flask under N_2, followed by addition of dry CH_2Cl_2 (200 mL). The slurry mixture was stirred magnetically at room temperature under N_2. DMAP (1.08 g, 8.8 mmol; 15 mol%) was added followed by slow addition of acetic anhydride (15.8 mL, 2.8 equivs. total) by syringe pump over a period of 8.5 h. A further portion of DMAP (0.36 g, 2.9 mmol) was added at 7.75 h (Note 2, below).
2. The reaction progress was monitored closely by HPLC (Note 3, below).
3. The reaction was quenched after 11 h by addition of water (5 mL) and the mixture stored at -20°C before workup. The mixture was filtered through a Celite pad to remove

insolubles and the Celite pad washed with CH_2Cl_2. The filtrate was then washed with 5% HCl (100 mL), H_2O (50 mL), satd. $NaHCO_3$ (3 x 100mL), and satd. NaCl(100mL), dried (Na_2SO_4), and filtered. The filtrate was then concentrated (~150 mL removed), EtOAc (100 mL) added and further concentrated to ~60 mL

4. With rapid stirring hexanes (420 mL) was added over a period of 5 min. The precipitated product was collected by filtration, washed with hexanes (100 mL) and dried under vacuum to yield a white solid (17.4 g, 81.2%) (Note 4, 5, below).

Notes

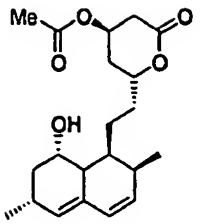
1. The triol acid was determined to be 77.5% pure by ^1H NMR assay with toluic acid as an internal standard; the rest of the material is precipitated protein/lyophilization material.
2. The rate of addition of acetic anhydride and DMAP are shown in Table 1

Table 1. The sequence of DMAP and acetic anhydride addition.

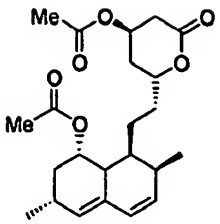
Time	0min	0-30min	3.5-4hr	6-6.5hr	7.75hr	8.5hr
DMAP (g)	1.083				0.36	
DMAP (mol%)	15				5	
Acetic anhydride (ml)	2	9.2	1.68	1.2		1.68
Acetic anhydride (eq.)	0.36	1.64	0.30	0.21		0.30

3. Samples were analyzed on a Waters 1100 Series HPLC, using a Zorbax SB-Phenyl column (4.6 x 75 mm) (40% MeCN/0.5% AcOH gradient; 1 ml/min; RT; 238 nm). The gradient and elution order were as follows:

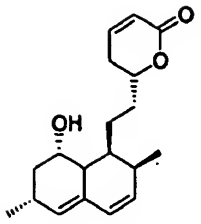
Time min	MeCN	0.5% AcOH	Component	Rt
0	37.5	62.5	Triol Acid	1.2
8	37.5	62.5	Diol Lactone	3.2
8.1	60	40	Elimination Product	7.5
12	60	40	4-Acetyl lactone	8.1
12.1	37.5	62.5	Diacetate	10.7



4-Acetyl Lactone



Diacetate



Elimination Product

4. A further 2.20 g (10.3%) of acetyllactone remained in the mother liquors, for a total yield of 91.5%.
5. HPLC area% showed: Diol lactone, 0.8%; 4-Acetyllactone, 98.5%; 4,8-Diacetate, 0.2%, Elimination, 0.6%.

B. DIRECT ACETYLATION OF TRIOL ACID (37 G SCALE) (2516-55)

1. The reaction was carried out as described above using crude triol acid (48.43 g, 111 mmol) (77.45% pure) (Note 1, below) and DMAP (2.30g, 18.8 mmol; 15mol%) in anhydrous CH_2Cl_2 (375mL). The reaction slurry was stirred magnetically at room temperature under N_2, and acetic anhydride (34.6 mL, 3.3 equivs.) was slowly added by syringe pump (Note 2, below).
2. Into a 1-L dry flask under N_2, triol acid (2287-40, 48.43g, 77.45%) was charged followed by sequential
3. The reaction was quenched after 8 h by addition of water (5 mL), stirred for 10 min, and the mixture stored at -20°C before workup. The mixture was filtered through a Celite pad to remove insolubles and the Celite pad washed with CH_2Cl_2. The filtrate was then washed with 5% HCl (175 mL), H_2O (50 mL), satd. $NaHCO_3$ (2 x 175mL, 100 mL), and satd. NaCl(175mL), dried (Na_2SO_4), and filtered. The filtrate was concentrated (300 mL removed), EtOAc (200 mL) added and concentrated to ~110 mL
4. With rapid stirring hexanes (450 mL) was added over a period of 5 min. The precipitated product was collected by filtration, washed with hexanes (50 mL) and dried under vacuum to yield a white solid (31.5 g, 78.4%) (Note 3, 4, below).

Notes

1. The triol acid was determined to be 77.5% pure by ^{1}H NMR assay with toluic acid as an internal standard; the rest of the material is precipitated protein/lyophilization material.
2. The rate of addition of acetic anhydride and DMAP are shown in Table 1

Table 1. The sequence of DMAP and acetic anhydride addition.

Time	0min	0-30min	0.5-2hr	2-4hr	5.5-7.5hr	8hr
DMAP (g)	2.301					
DMAP (mol%)	15					
Acetic anhydride (ml)	4.2	16.8	8.37	2.1	2.1	1.05
Acetic anhydride (eq.)	0.4	1.6	0.8	0.2	0.2	0.1

3. A further 3.4 g (8.5%) of acetyllactone remained in the mother liquors, for a total yield of 86.9%.
4. HPLC area% showed: Diol lactone, 1.4%; 4-Acetyllactone, 97.4%; 4,8-Diacetate, 0.3%, Elimination, 0.6%.

C. DIRECT ACETYLATION OF TRIOL ACID (150 G SCALE) (2516-60)

1. The reaction was carried out as described above using crude triol acid (154 g) (Note 1, below) and DMAP (6.8g, 55.7 mmol; 15mol%) in anhydrous CH_2Cl_2 (1 L). The reaction slurry was stirred mechanically under N_2, and acetic anhydride was slowly added by syringe pump (Note 2, below). The reaction was held at 15°C for an initial 1.5 h, then stirred at room temperature.
2. The reaction was quenched after 9.25 h by the addition of water (200 mL), stirred at room temperature for 20 min, then allowed stand overnight.
3. The reaction mixture was filtered through a pad of Celite, which was then washed with CH_2Cl_2 (2 x 250mL). The combined filtrates were sequentially washed with 5% HCl (500mL) and H_2O (500mL), and then concentrated (1.2L CH_2Cl_2 removed). EtOAc (500mL) was added to the residue and a further 400mL solvent was removed. The remaining solution was washed with satd. $NaHCO_3$ (500mL), then stirred with a $NaHCO_3/H_2O$ mixture (500mL satd. $NaHCO_3$, 500ml H_2O with a further 167.2g $NaHCO_3$ powder added in portions)(Note 3, below).

4. The two layers were separated slowly on standing and the organic layer was washed with NaCl (250mL). The organic layer was dried (Na_2SO_4), filtered and concentrated to ~500 mL
5. With rapid stirring, hexanes (3.5 L) were added to the residue over a period of 45 min. The precipitated solid was filtered and dried to yield a white solid (95 g, 70.7%) (Note 4, 5, below).

Notes

1. The crude triol acid was material isolated from the hydrolysis of 150 g lovastatin and carried forward.
2. The rate of addition of acetic anhydride is shown in the Table:

Table . The sequence of acetic anhydride addition

Time	0-30min	0.5-1.5hr	1.5-2.5hr	3.5-3.7hr	4.25	5hr	5.7hr
Acetic anhydride (mL)	70.13	28.05	10.5	7	14	14	14
Acetic anhydride (eq.)	2.0	0.8	0.3	0.2	0.4	0.4	0.4

3. Removal of all acetic and 2-methylbutyric acid is essential to prevent their re-introduction in the subsequent acylation reaction.
4. A further 10.1 g (7.5%) of acetyllactone remained in the mother liquors, which combined with ~0.16% product lost to the aqueous washes, represented a total yield of 78.4% from lovastatin.
5. HPLC area% showed: Diol lactone, 0.9%; 4-Acetyllactone, 98.7%; 4,8-Diacetate, 0.2%, Elimination, 0.1%.
6. ^{1}HNMR ($CDCl_3$) δ 0.90 (d, J = 6.94 Hz, 3 H), 1.19 (d, J = 7.57 Hz, 3 H), 1.27-1.41 (m, 1 H), 1.45-1.60 (m, 2 H), 1.76-1.95 (m, 6 H), 2.09 (s, 3 H), 2.10-2.13 (m, 1 H), 2.14-2.20 (m, 1 H), 2.32-2.41 (m, 1 H), 2.41-2.50 (m, 1 H), 2.67-2.75 (m, 1 H), 2.75-2.82 (m, 1 H), 4.23 (br s, 1 H), 4.54-4.63 (m, 1 H), 5.22-5.28 (m, 1 H), 5.53-5.58 (m, 1 H), 5.77-5.83 (m, 1 H), 5.99 (d, J = 9.46 Hz, 1 H); ^{13}CNMR ($CDCl_3$) δ 13.98, 21.07, 23.82, 24.19, 27.40, 30.82, 32.95, 33.39, 35.40, 35.83, 36.50, 38.77, 65.34, 65.61, 76.51, 128.51, 130.14, 131.29, 133.60, 168.90, 170.02.

D. DIRECT ACETYLATION OF TRIOL ACID (150 G SCALE) (2516-84)

a. Crude triol acid (151.21 g from 150 g lovastatin) was charged to a 2-L dry flask followed by addition of CH_2Cl_2 (1.0 L). The slurry was agitated by an overhead mechanical stirrer and left overnight at ambient temperature.

b. DMAP(6.8g, 15mol% based on 150g lovastatin) was added in one portion, followed by addition of acetic anhydride (157.6ml, 4.5 equiv.) over a 20 min period. The reaction was monitored by HPLC.

c. The reaction was quenched after 3.5 h by addition of water (100ml) and was stirred for an additional 3 h at ambient temperature. The reaction mixture was filtered through a Whatman #1 filter paper and the filter cake was washed with CH_2Cl_2 (2 x 250ml).

d. The CH_2Cl_2 was sequentially washed with 5% HCl (500ml) and H_2O (500ml), and then the organic layer was concentrated to 400ml and diluted with EtOAc (500ml). This solution was stirred with satd. $NaHCO_3$ (500ml), with additional $NaHCO_3$ (60g) being added to neutralize acetic acid. The organic layer was washed with satd. NaCl (500ml), dried (Na_2SO_4), and filtered. The filtrate was concentrated to ~100 mL. With stirring, hexanes (500 ml) was added rapidly to the residue. The precipitated solid was filtered and dried to yield a white solid (112.6g, 83.4%) (Note 1, 2, below).

Notes

1. A further 7.6 g (5.7%) of 4-acetyllactone remained in the mother liquors, representing a total yield of 89.1% from lovastatin.
2. HPLC area% showed: Diol lactone, 0.9%; 4-Acetyllactone, 99.0%; 4,8-Diacetate, 0.45%, Elimination, 0.53%.

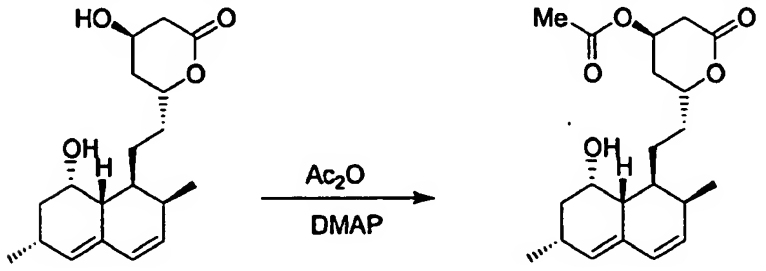
E. DIRECT ACETYLATION OF TRIOL ACID (150 G SCALE) (2516-87)

1. Crude triol acid (158.4 g from 150 g lovastatin) was charged to a 2-L dry flask followed by addition of CH_2Cl_2 (625 ml). The slurry was agitated by an overhead mechanical stirrer and left overnight at ambient temperature.
2. DMAP(6.8g, 15mol% based on 150g lovastatin) was added in one portion, followed by addition of acetic anhydride (122.6ml, 3.5 equiv.) over a 17 min period. The reaction was monitored by HPLC. A further portion of acetic anhydride (35 ml, 1.0 equiv.) was added at 2.5 h followed by addition of Et_3N (25.8 ml, 0.5 equiv.) at 3.5 h (Note 1, below).
3. The reaction was terminated after 6.3 h, and submitted to the same extractive workup as described previously. This time addition of hexanes precipitated the product as large chunks. The solid was redissolved in CH_2Cl_2 (300 ml) and EtOAc (300 ml), and concentrated to ~130 mL. Addition of hexanes (650 ml) precipitated the product, which was collected and dried to give a white solid (107.24g, 79.8%) (Note 2, 3, below).

Notes

1. The reaction stopped at ~60% conversion and Et_3N was added to assist acetylation.
2. A further 10.7 g (8.0%) of 4-acetyllactone remained in the mother liquors, representing a total yield of 87.8% from lovastatin.
3. HPLC area % showed: Diol lactone, 0.6%; 4-Acetyllactone, 97.9%; 4,8-Diacetate, 0.6%, Elimination, 0.9%.

F. ACETYLATION DIOL LACTONE WITH ACETIC ANHYDRIDE

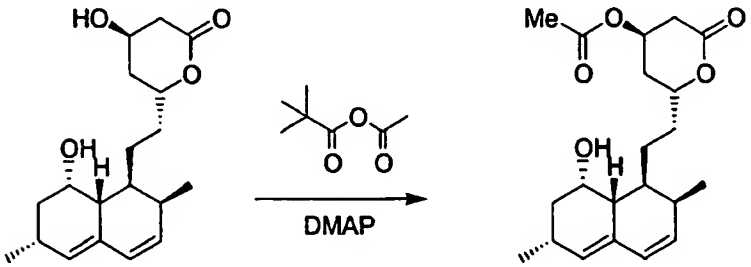


$C_{19}H_{28}O_4$
Mol. Wt.: 320.42

$C_{21}H_{30}O_5$
Mol. Wt.: 362.46

1. Diol lactone (38.57 g, 120 mmol) (Note 1) and DMAP (2.21 g, 18.1 mmol; 0.15 equiv.) were dissolved in anhydrous CH_2Cl_2 (240 mL) in a dry 3-neck flask. The mixture was stirred under N_2 and cooled to 0°C *via* external ice-water bath.
2. Acetic anhydride (11.4 mL, 122 mmol, 1.02 equiv.) was added in two portions. The first portion (2.4 mL) was added in one shot, followed by the slow addition of the second portion (9 mL) by syringe pump over a period of 30 min. The reaction mixture was stirred at 0°C for 4 hours, for another 30min with the temperature gradually raised to 10°C by removal of the cold bath.
3. The reaction was quenched by adding water (60 mL) and stirred for 10 min at ambient temperature. The mixture was then washed sequentially with 10% HCl (60 mL), water (120 mL), satd. $NaHCO_3$ (120 mL) and satd. NaCl (120 mL). The organic layer was collected and dried (Na_2SO_4), and evaporated to yield a slightly yellowish glassy solid (41.9 g, 96.1%) (Note 2).

G. ACETYLATION OF DIOL LACTONE WITH PIVALIC/ACETIC MIXED ANHYDRIDE



$C_{19}H_{28}O_4$
Mol. Wt.: 320.42

$C_{21}H_{30}O_5$
Mol. Wt.: 362.46

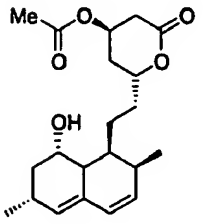
1. Diol lactone (10.0 g, 31.3 mmol,) and DMAP (0.57 g, 4.7 mmol; 0.15 equiv.) were dissolved in anhydrous CH_2Cl_2 (62.5 mL), and the mixture stirred under N_2 and cooled to 0°C.
2. Pivalic/acetic mixed anhydride (4.95, 34.4 mmol; 1.1 equiv.) was added in two portions. The first portion (2 mL) was added in one shot, followed by the slow addition of the remainder by syringe pump over 20min. The reaction mixture was stirred 0°C for 30min, then at ambient temperature for 1.5 hours.

3. The reaction was quenched by addition of water (30 mL) and stirred for 10 min at ambient temperature. The mixture was washed sequentially with 5% HCl (30 mL), satd. $NaHCO_3$ (32 mL) and satd. NaCl (32 mL). The organic layer was collected, dried (Na_2SO_4), filtered and evaporated to yield a slightly yellowish solid (10.8 g, 95.5%) (Note 3).

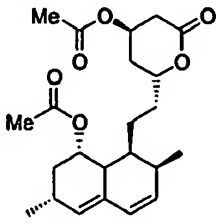
Notes

4. The starting diol lactone was 98.2% area on HPLC with 1.8% elimination product.
5. On the basis of HPLC area%, the crude product contains 4-acetyl lactone (92.3%), diol lactone (3.6%), 4, 8'-diacetyl lactone (1.5%), elimination product (2.6%).
6. On the basis of HPLC area%, the crude product contains 4-acetyl lactone (95.3%), diol lactone (2.1%), 4, 8'-diacetyl lactone (1.2%), elimination product (1.4%).
7. Samples were analyzed on a Waters 1100 Series HPLC, using a Zorbax SB-Phenyl column (4.6 x 75 mm) (40% MeCN/0.5% AcOH gradient; 1 ml/min; RT; 238 nm). The gradient and elution order were as follows:

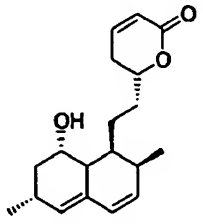
Time min	MeCN	0.5% AcOH	Component	Rt
0	37.5	62.5	Triol Acid	1.2
8	37.5	62.5	Diol Lactone	3.2
8.1	60	40	Elimination Product	7.5
12	60	40	4-Acetyl lactone	8.1
12.1	37.5	62.5	Diacetate	10.7



4-Acetyl Lactone



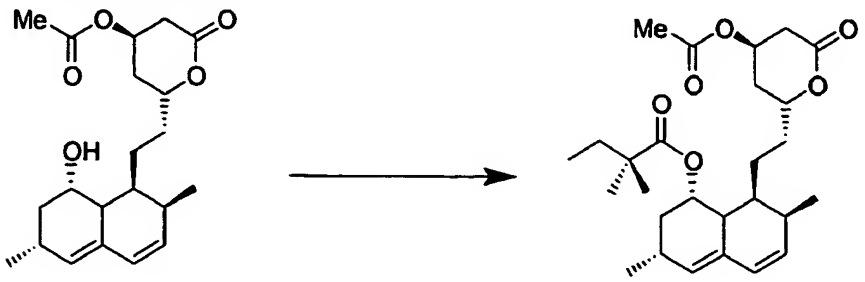
Diacetate



Elimination Product

Example 4: SYNTHESIS OF 4-ACETYLSIMVASTATIN

The following example describes an exemplary protocol of the invention for the synthesis of 4-acetyl-simvastatin.



$C_{21}H_{30}O_5$
Mol. Wt.: 362.46

$C_{27}H_{40}O_6$
Mol. Wt.: 460.60

A. Boron Trifluoride Etherate Catalysis (2719-95)

1. 4-Acetyllactone (110 g, 0.3 mol) was dried overnight under vacuum (0.1 torr) in a 2-neck 2L flask (Note1).
2. The dried starting material was dissolved in anhydrous CH_2Cl (875 mL) under N_2 at room temperature.
3. The catalyst was prepared as follows. In a glove bag under N_2, 2,2-dimethylbutyric anhydride (7.1 mL, 30.3 mmol) was added to anhydrous acetonitrile (125 mL), followed by the addition of freshly opened $BF_3.OEt_2$ (3.1 mL, 24.3 mmol; 8 mol%) (Note 2,3).
4. 2,2-Dimethylbutyric anhydride (78 mL, 0.33 mol; 1.1 equiv.) was added to the solution of 4-acetyllactone and the mixture was heated to 40°C for 10 minutes (Note 4). The MeCN solution of $BF_3.OEt_2$ was then added via cannula. (Note 5). The reaction was shielded from light, stirred at 40°C and monitored by HPLC.
5. After 5.5 h the reaction was judged complete and the reaction was cooled to 5°C in an ice bath. Satd. $NaHCO_3$ (250 mL) was added with vigorous stirring. The aqueous layer was separated and extracted with CH_2Cl_2 (200 mL).
6. The organic extracts were combined, dried (Na_2SO_4), filtered and concentrated under reduced pressure. MeOH (200 mL) was added to the concentrate (Note 6); removal of more MeOH results in precipitation of 4-acetylsimvastatin. The off-white solid was filtered, washed with cold MeOH (100 mL) and dried under vacuum (92.8 g).

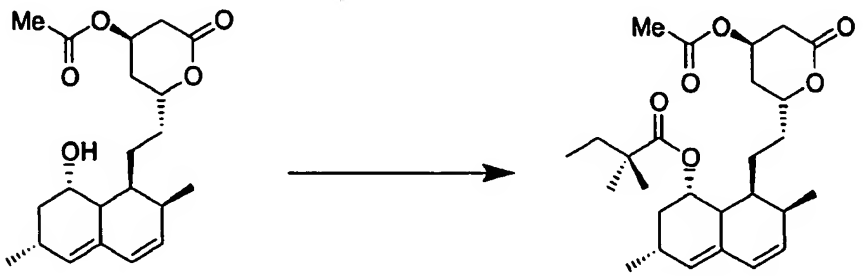
7. The mother liquors were concentrated to about half volume and cooled at -10°C overnight. A second crop if product (17.2 g) was collected by filtration and dried (Note 7).
8. The HPLC profile is shown in the Table.

Peak Identity	Retention Time Min	Area %
4-Acetyllactone	1.73	0.06
4,8-Bisacetate	2.37	0.80
Simvastatin	2.52	0.04
Unknown	3.52	0.03
4-Acetyl Lovastatin	3.80	0.80
4-Acetyl Simvastatin	4.59	97.78
Anhydrosimvastatin	5.47	0.31
4-Simvastain-8-Lovastatin	8.30	0.03
Bis-Simvastatin	9.78	0.10
Total Area		99.95

Notes

1. The starting material should be ground to a powder to facilitate the removal of acetic acid which mat be entrained in large chunks. Residual acetic will result in formation of the 4,8-diacetate. Drying at elevated temperature under vacuum may cause decomposition. 4-Acetyllactone turned yellowish when dried at 40°C under vacuum.
2. Since the reaction is sensitive to the presence of moisture, excess anhydride was initially added to the acetonitrile to scavenge any residual water. Preheating the anhydride and acetyllactone scavenges water from the reaction vessel.
3. Freshly opened $BF_3.OEt_2$ should be used for the reaction; reagent that has been opened previously can result in slow, or even, no reaction.
4. The solution must be cooled down during addition of catalyst, otherwise aromatic byproduct is formed.
5. The CH_2Cl_2 /MeCN ratio was 7:1. Typically the ratio is between 6:1 and 9:1. The reaction is faster in MeCN but the product is formed with a less desirable impurity profile.

6. MeOH should be added before crude product solidifies, otherwise it is difficult to re-dissolve it in MeOH. Dissolving solid product in hot methanol caused decomposition and thus gave lower yield.
7. Total solid product was 110 g (78.7%). The final mother liquors were evaporated to dryness and the residue was assayed versus a working standard and shown to contain a further 9.02 g (6.8%) of product. A further ~2% product remained in the aqueous washes.



$C_{21}H_{30}O_5$
Mol. Wt.: 362.46

$C_{27}H_{40}O_6$
Mol. Wt.: 460.60

B. Synthesis of 4-Acetylsimvastatin (2958-12)

Prepared as described above.

4-Acetyllactone (2516-84) (111.6 g; 91%).

1st crop: 86.2g

2nd crop: 11.6g

Total: 97.8g , 75.8%.

Assay:

^{1}H-NMR 99.8% (versus toluic acid as internal standard)

HPLC 98.1% (versus working standard of 4-acetylsimvastatin)

The aqueous washes contained ~1.9% and a further ~7% remained in residues for a total yield of 84.7%.

The HPLC profile is shown in the Table.

Peak Identity	Retention Time Min	Area %
4-Acetyllactone	1.73	0.06

4,8-Bisacetate	2.37	1.42
Simvastatin	2.52	~
Unknown	3.52	~
4-Acetyl Lovastatin	3.80	0.20
4-Acetyl Simvastatin	4.59	97.76
Anhydrosimvastatin	5.47	0.50
4-Simvastain-8-Lovastatin	8.30	0.06
BisSimvastatin	9.78	~
Total Area		100

C. Synthesis of 4-Acetylsimvastatin (2958-16)

Prepared as described above.

4-Acetyllactone (2516-87) (107 g; 96%).

1st crop: 90.4g

2nd crop: 12.7g

Total: 97.8g , 79.3%.

Assay:

^{1}H-NMR 99.2% (versus toluic acid as internal standard)

HPLC 96.8% (versus working standard of 4-acetylsimvastatin)

The aqueous washes contained ~1.8% and a further ~7% remained in residues for a total yield of 88.1%.

The HPLC profile is shown in the Table.

Peak Identity	Retention Time Min	Area %
4-Acetyllactone	1.73	0.04
4,8-Bisacetate	2.37	2.20
Simvastatin	2.52	~
Unknown	3.52	~
4-Acetyl Lovastatin	3.80	0.31
4-Acetyl Simvastatin	4.59	97.00
Anhydrosimvastatin	5.47	0.35
4-Simvastain-8-Lovastatin	8.30	0.02
Bis-Simvastatin	9.78	0.08
Total Area		100

D. Pyridine/DMAP Method

1. 4-Acetyllactone (2.6 g, 7.2 mmol) was dried under vacuum overnight at room temperature, then dissolved in anhydrous pyridine (6.0 mL) with stirring at room temperature under nitrogen. A solution of DMAP (176 mg, 0.2 equiv.) in 1.5 mL anhydrous pyridine was added and the mixture cooled in an ice bath.
2. 2,2-Dimethylbutyryl chloride (7.72 g, 8equiv.) was added dropwise over 15 minutes using a syringe pump. The mixture was stirred at 0°C for about one hour, then at room temperature for one hour.
3. The reaction mixture was heated at 40°C under nitrogen and reaction was monitored by HPLC. After the 4-acetyllactone was consumed (2 days), the pyridine was removed by rotary evaporation. The residue was partitioned between EtOAc (20 mL) and saturated NaCl (20 mL). The organic layer was dried (Na_2SO_4), filtered and evaporated to give the crude product (96.5%) (Notes1,2).

E. $Cu(OTf)_2$/Anhydride Method

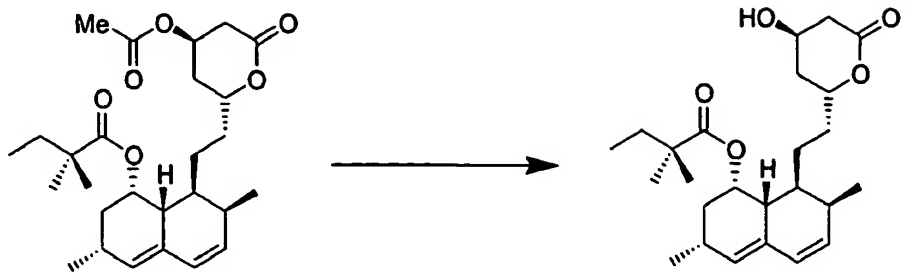
1. 10.0g of 4-Acetyllactone (10.0 g, 27.6 mmol) was dried under vacuum at room temperature for 1hr, then dissolved in anhydrous CH_2Cl_2 (60 mL) and stirred under nitrogen.
2. Meanwhile, a solution of $Cu(OTf)_2$ (0.5g 5 mol%) and 2,2-dimethylbutyric anhydride (7.15 mL, 30.5 mmol) in anhydrous MeCN (7.0 mL) was prepared and stirred at room temperature inside a sealed flask.
3. The lactone solution was cooled to 15°C. The solution of $Cu(OTf)_2$ and 2,2-dimethyl butyryl anhydride was added dropwise using syringe pump. The reaction was monitored by HPLC and judged complete within 3.0 hours.
4. The reaction was quenched with water (20 mL) and partitioned between CH2Cl2 (100 mL) and satd. NaCl (100 mL). The organic layer was then stirred for 10 minutes with a mixture of 1M malic acid (50 mL) and satd. NaCl (50 mL), then satd. NaCl (100 mL). The organic layer was dried (Na2SO4), filtered and evaporated to yield the crudeproduct (12.8g >100% yield by weight) (Note 3,4).

Notes

1. The product distribution by HPLC area% was: 4-acetylsimvastatin (79.5%), elimination product (12%), bissimvastatin (2%), unidentified impurity (6.5%).

2. 4-Acetylsimvastatin was isolated in 43% after column chromatography. 4-Acyl simvastatin is believed to possess limited stability to SiO_2 chromatography.
3. The product distribution by HPLC area% was: 4-acetylsimvastatin (92.5%), elimination product (2.7%), bissimvastatin (1.7%), unidentified impurity (3.1%).
4. 4-Acetylsimvastatin was isolated in 61% after column chromatography.

Hydrolysis of 4-Acetylsimvastatin by SEQ ID NO:4



$C_{27}H_{40}O_6$
Mol. Wt.: 460.60

$C_{25}H_{38}O_5$
Mol. Wt.: 418.57

1. A solution of 4-acetylsimvastatin (3.68 g, 8 mmol) in MeOH (2 mL) was added to a mixture of 4M Tris buffer (0.1 mL) in water (9.9 mL) in a 25 mL 3-neck flask. The slurry was stirred vigorously (both magnetic and overhead stirring) and heated to 50°C.
2. SEQ ID NO:4 (1 g lyophilized material) was dissolved in water (8 mL) and added to the reaction mixture.
3. pH was maintained at 6.75 using a DASGIP FEDBATCH -pro® system, by addition of 10% NH_3 in water, and the reaction temperature maintained at 50°C using a heated water bath.
4. Once the reaction had reached 75% conversion, toluene (4 mL) was added in order to solubilize the product and remaining starting material.
5. Aliquots (20 µL quenched in 980 µL MeOH) were taken periodically to monitor progress of the reaction by HPLC (Note 1, below).

When judged complete, the reaction mixture was clarified by centrifugation (45000 x *g*, 4°C, 25 min) to give a toluene top layer, an aqueous clarified layer and a compressed solid pellet. The clarified aqueous centrifugate was adjusted to pH 2.5 with HCl. A flocculent precipitate was observed. This mixture was clarified by centrifugation (45000 x *g*, 4°C, 25 min), resulting in another small pellet.

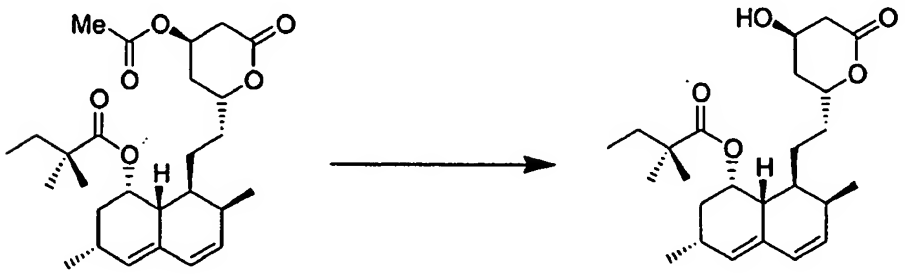
6. Upon examination of each fraction by HPLC, the simvastatin is concentrated in the organic phase and pelleted materials. The pellets were extracted by dichloromethane (100mL) and the resulting emulsion was separated by centrifugation (45000 x *g*, 4C, 25 min). The CH_2Cl2 layers were combined, dried (Na_2SO_4) and evaporated to give a yellow oil (3.05g, 91%) (Note 2, below).

Notes

1. Samples were analyzed on a Waters 1100 Series HPLC, using a Zorbax SB-Phenyl column (4.6 x 75 mm) (45% MeCN/0.1% H_3PO_4 gradient; 1 ml/min; RT; 238 nm). The gradient and elution order were as follows:

Time min	MeCN	0.1% H_3PO_4	Component	Rt
0	45	55	Simvastatin Acid	4.7
10	45	55	Simvastatin	9
18	85	15	Acetyl Simvastatin	15.2
19	85	15	Eliminated Simvastatin	15.5
12.1	37.5	62.5		

HYDROLYSIS OF 4-ACETYLSIMVASTATIN BY SEQ ID NO:4



$C_{27}H_{40}O_6$
Mol. Wt.: 460.60

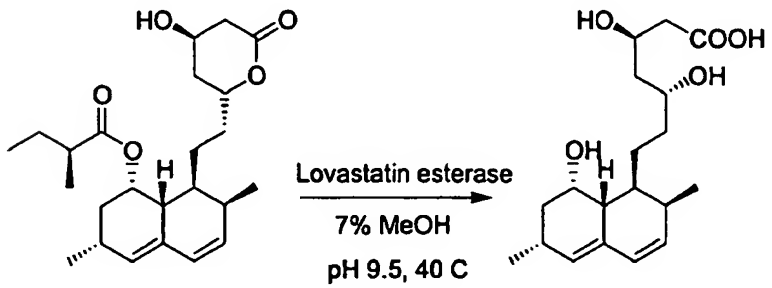
$C_{25}H_{38}O_5$
Mol. Wt.: 418.57

1. A mixture of 4-acetylsimvastatin (96.6 g, 0.21 mol) and SEQ ID NO:4 (20 g) was suspended in 10% MeOH (1 L) in a 2-L round bottom flask equipped with a magnetic stir-bar and an overhead stirrer. The mixture was stirred vigorously and maintained at 60°C in a heated water bath.
2. pH was maintained at 7.5 using a DASGIP FEDBATCH-pro® system, by addition of 10% NH_3 in water. The reaction was monitored by HPLC.

3. After 24 h, the reaction mixture was transferred into 4 x 250 mL centrifuge bottles and centrifuged at 10,000 rpm at 4°C for 15 min. The supernatant was decanted and discarded. The pellets were resuspended in water (4 x 250 mL) and centrifuged as before. Again the supernatant was decanted and discarded.
4. The centrifuge pellets were transferred to a sintered glass funnel and excess water removed. The centrifuge bottles were rinsed with acetone (2 x 150 mL) which was transferred to the funnel. Celite (10 g) was added to the funnel, the mixture triturated and then sucked dry.
5. The residue on the funnel was washed with CH_2Cl_2 (5 x 200 ml), triturating after each portion and adding further Celite as necessary.
6. The combined washings were washed with satd. NaCl (100 ml) and the aqueous layer discarded. The organic layer was dried (Na_2SO_4), filtered, and the solvent exchanged for toluene (200 ml).
7. Hexanes (600 ml) was added with stirring to the toluene solution; precipitation started after ~300 ml had been added. The precipitated product was filtered and dried to yield a white solid (69.9 g, 79.7%)
8. The mother liquors were cooled to -20°C overnight and a second crop of simvastatin was collected (3.5 g, 4.0%).

Example 5: Synthesis of Simvastatin from Lovastatin

STEP 1: LOVASTATIN HYDROLYSIS



Lovastatin **Triol Acid**

Having identified a novel lovastatin esterase (having a sequenced as set forth in SEQ ID NO:4 and subsequent subclones), efforts focused upon producing a scaleable enzymatic hydrolysis process. Among the required parameters for the proposed simvastatin process was that the enzymatic reaction be run at 20% w/v substrate (~0.5M). Initial screening and confirmatory reactions were carried out using lovastatin acid, because of its high aqueous solubility. Reactions using lovastatin were much slower because of the lower solubility of lovastatin in water, especially at lower pH's (7-8) and high substrate loading.

Lack of solubility was overcome by first chemically opening the lactone ring in-situ. Thus a suspension of lovastatin in MeOH/water (final reaction concentration 7-10% MeOH) was treated with 1 equivalent of NaOH and the mixture stirred for a couple of hours until the lovastatin had been converted to the more soluble lovastatin acid. When ring-opening was complete, the pH of the reaction mixture was adjusted to pH 9.5 before addition of the enzyme, although adjustment was not necessary in many cases as the pH fell to an acceptable value as the ring opening proceeded.

The enzymatic reaction was initiated by addition of a solution of the reconstituted enzyme. The mixture was then stirred at 35-40°C, with the pH being held constant at pH 9.5 by automatic addition of 10-30% NH_4OH. Under these conditions > 98% conversion of lovastatin to triol acid was generally obtained in 48 h. The reaction slows down considerably towards completion. The results for a series of large scale hydrolyses are gathered in Table 1.

Table 1. Hydrolysis of Lovastatin

Run	Substrate g	Enzyme Lot	Enzyme g	Time h	Triol Acid %	Lovastatin Acid %
1	100	SEQ ID NO:4	22	48	98.7	0.5
2	150	SEQ ID NO:4-1	25	86	98.8	1.2
3	150	SEQ ID NO:4-1	25	108	99.1	0.9
4	10	SEQ ID NO:4-1	2.2	41	98.7	1.0
5	150	SEQ ID NO:4-2	30	46	99.1	0.9
				52	99.5	0.5

6	150	SEQ ID NO:4-2	30	48.5	98.6	1.4
				64	99.5	0.5

Runs 2 and 3 showed abnormally long reaction times. In these two cases, the lovastatin lactone opening was carried out using a large excess of NaOH and required addition of HCl to return the pH to a suitable range for the enzymatic reaction. It had previously been observed that high salt concentrations had a deleterious effect on the enzymatic hydrolysis.

Furthermore, due to limited availability at the time, the initial enzyme charge (11% w/w) was less than used previously; further portions of enzyme were added to bring the final enzyme charge to 17% w/w.

The reaction was terminated by diluting the reaction mixture with water and then acidifying the mixture to pH~2. Under these conditions the triol acid, denatured protein and other media/cell components precipitated from solution.

For initial small scale, dilute reactions, this mixture was subjected to continuous liquid extraction with refluxing iPrOAc. Under these conditions the lactonization of triol acid occurred and the diol lactone could be easily obtained by precipitation from the concentrated iPrOAc extract.

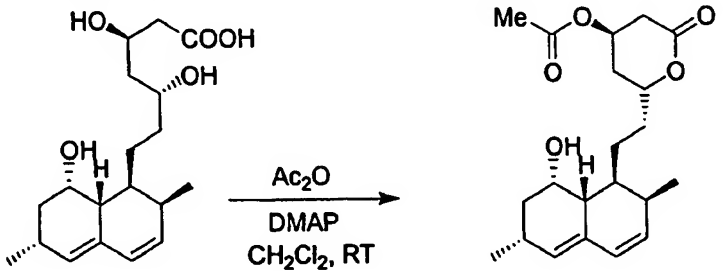
For larger scale reactions the precipitated triol acid/denatured protein mixture was isolated by filtration and, while still damp, the filter cake was suspended in iPrOAc and subjected to azeotropic distillation to effect lactonization. The insoluble, denatured protein/cell components were removed by filtration and the diol lactone isolated by concentration and precipitation. This procedure worked well on a 10-30 g scale to generate the diol lactone without purification of the triol acid. However as the scale of the reaction increased (50-100 g), the azeoptropic distillation required longer reflux periods in more concentrated solutions to effect lactonization. The yield of diol lactone isolated under these conditions was diminished, and the product was contaminated with increasing quantities of yellow oil, presumably caused by polymerization of the triol acid or diol lactone.

At >100 g scale in the laboratory, the most convenient workup was to dilute and acidify the enzymatic reaction mixture. The insoluble materials were collected by filtration and this damp filter cake was dried; initially lyophilization was used for drying, but

for more recent runs the filter cake has been dried in a vacuum oven at 30-40°C. Assaying the crude product (^{1}H NMR in the presence of an internal standard) indicated that it contained ~78% triol acid, the rest of the material being denatured protein, cell and media components.

After filtration the filtrate could be extracted with EtOAc to recover a further ~2% of product. This material could be isolated, either as the triol acid or lactonized (7 mM $MeSO_3H$) to the diol lactone, and added to the next step.

STEP 2: ACETYLATION



Triol Acid **4-Acetyllactone**

In its early phase, the process required the acetylation of diol lactone to form 4-acetlylactone. Since the use of acetic anhydride for the acetylation of diol lactone was already claimed in an existing patent, the use of a mixed anhydride was examined. The acetic/pivalic mixed anhydride was prepared and compared to acetic anhydride. The mixed anhydride offered no advantages over acetic anhydride; similar ratios of 4-acetyllactone and diacetate were obtained, and byproducts resulting from introduction of the pivalate were observed in the subsequent acylation step.

Subsequent changes to the process, namely (i) the direct acylation from triol acid to 4-acetyllactone and (ii) improved conditions for the introduction of the dimethylbutyrate side-chain (see later), improved the process and provided sufficient differentiation to allow us to use acetic anhydride in the acetylation step.

The crude product from the lovastatin hydrolysis step contains triol acid and denatured protein and cell/media components. This crude material was suspended in CH_2Cl_2 (10-15% w/v) and treated with acetic anhydride (3 equivs.) in the presence of DMAP (0.15 equivs.). Initially the acetic anhydride was added portion-wise; the first equivalent added to

effect closure of the lactone ring, followed by portion-wise addition of the rest of the acetic anhydride. This conservative approach was taken to minimize formation of diacetate. However subsequent studies have showed that acetylation of the 8-position of 4-acetyllactone is slow and can be reasonably controlled. Therefore more recent runs have added all three equivalents of anhydride to the reaction at the beginning. The reaction is monitored by HPLC and is typically terminated when <2% diol lactone remains; at this point <2% of diacetate is formed. Some elimination product may be formed, especially if the reaction is stirred for excessively long periods.

After completion the reaction is quenched by the addition of water, and the insoluble materials are removed by filtering through a Celite pad. This pad is washed with CH_2Cl_2 and the combined filtrates are washed with dilute acid (to remove DMAP) and with satd. $NaHCO_3$ to remove acetic acid. On large scale it was found more convenient after the acid wash, to carry out a solvent exchange for EtOAc to facilitate the subsequent washing with base. It is important that all organic acids (either acetic acid or 2-methylbutyric acid from the original lovastatin) be removed at this stage; failure to do so will result in preferential introduction of an acetate or methylbutyrate side-chain in the following step when the simvastatin side-chain is introduced.

After base extraction, the solution is dried, filtered and concentrated. Addition of hexanes then leads to the precipitation of 4-acetyllactone as a white solid. The yields and product profiles for several larger runs are collected in Table 2.

Table 2. Direct acetylation of triol acid to 4-acetyllactone

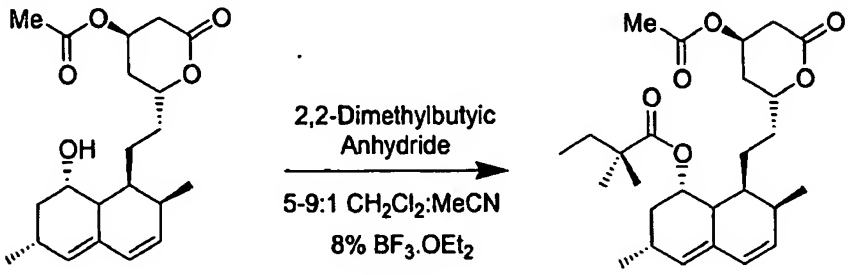
	Run	Triol Acid g	Time h	Yield %	Diol Lactone %	4-AcLactone %	DiOAc %	Elimination %
2516-51	1	26	11	81.2 (91.5)[1]	0.8	98.5	0.2	0.6
2516-55	2	48	8	78.4 (86.9)[1]	1.4	97.4	0.3	0.6
2516-60	3	154	9.25	70.7 (78.2)[2]	0.9	98.7	0.17	0.11
2516-64	4	147	9.2	78.1 (84.1)[2]	0.6	97.6	0.5	0.5[3]
2516-84	5	151	3.5	83.8 (89.4)[1]	0	99.0	0.5	0.5
2516-87	6	158	6.3	79.8 (87.8)[1]	0.6	97.9	0.6	0.9

[1]Values in parentheses include unrecovered product in the mother liquors

[2] Values in parentheses include a recovered second crop of product

[3] Also contains 0.24% 4-AcLovastatin, and 0.5% of an unknown impurity at 4.0 min

STEP 3: ACYLATION



4-Acetyllactone **4-Acetylsimvastatin**

Catalyst identification

Reported conditions for the introduction of the simvastatin side-chain were not suitable for process scale-up. The reaction (i) is run in neat pyridine, (ii) uses up to 8 equivalents of 2,2-dimethylbutyryl chloride, and (iii) requires several days at elevated temperature. In our hands the product isolated from such reaction conditions was obtained in low yield and was of poor quality (elimination of the 2-acetoxy group was a major problem). Alternative solvents/bases did not improve the reaction.

Considerable improvement was achieved by switching to a Lewis acid-catalyzed reaction using dimethylbutyric anhydride as the acylating agent. Following literature precedent, bismuth triflate ($Bi(OTf)_3$) was examined; $Bi(OTf)_3$ has been reported as an effective catalyst for the pivaloylation of alcohols. The reaction was much cleaner than the pyridine route. However, $Bi(OTf)_3$ is not commercially available, its preparation is covered under existing intellectual property, and bismuth residues were difficult to remove from the product. Copper triflate ($Cu(OTf)_2$), which is commercially available, also worked well, giving good yields of product with only 10% load of catalyst and 1.05 equivalents of dimethylbutyric anhydride at room temperature. In this case removal of copper salts was a problem.

At this time, we had already surveyed a series of Lewis acids for their ability to catalyze the regioselective acylation of diol lactone at the 8-position to give simvastatin directly. Of the >20 Lewis acids surveyed, activity was seen with the triflate salts of bismuth,

copper, scandium, indium, aluminum, and with TMSOTf and $BF_3.OEt_2$. The triflate salts of Li, Mg, Zn, La, Pr, Sm, Yb were not active under the same conditions, nor were pyridinium or imizdazolium triflate, nor the acetate salts of Bi, In, or Sr.

$BF_3.OEt_2$ was an attractive catalyst for the acylation of 4-acetyllactone since it is cheaply available. Various other adducts of boron trifluoride were tested as acylation catalysts. Neither the THF adduct nor the dimethylamine adduct of BF_3 were suitable Lewis catalysts. Activity was seen with other commercially available BF_3.solvates but, since they offered no advantage over $BF_3.OEt_2$, further optimization was carried out with the etherate.

Optimization of conditions

A range of solvents and conditions were tested for both the triflate and BF_3 etherate-catalyzed acylation of 4-acetyllactone, as illustrated in Figure 1. The best results were obtained in CH_2Cl_2, MeCN, dichloroethane, or mixtures thereof. The results of several $BF_3.OEt_2$ catalyzed acylations are collected Table 3, illustrated in Figure 2.

The reaction was faster with a higher ratio of MeCN present but gave a poorer yield (Cf. runs 1,3). Better results were observed using fresh $BF_3.OEt_2$ (Cf. runs 1,2,6); previously opened bottles (run 2) and prealiquoted stock solutions (run 6) of $BF_3.OEt_2$ in MeCN gave poorer results. A minimum catalyst concentration was required; 4 mol% catalyst gave incomplete reaction (run 4).

In all reactions, a range of minor impurities could be seen. Some of these, e.g., the diacetate or 4-acetyllovastatin were present in the starting 4-acetyllactone, or were the direct result of impurities in the starting material, e.g., bissimvastatin which is formed from diol lactone. The levels of most of these impurities could be significantly reduced by precipitating the crude product from aqueous MeOH; Table 4 shows the impurity profile for the product of a 12 g acylation reaction, before and after precipitation, as illustrated in Figure 3. The yields for a series of reactions at the 20-100 g scale are shown in Table 5; isolated yields as well as the location and estimated amounts of the remaining product are indicated.

Table 5. Acylation of 4-acetyllactone: Results

Run	4-Ac Lactone[1] g	Time h	Isolated solid[2] g	Aqueous[3] g	Residue[4] g %	Total Yield %

			%	%		
1	21.0	3	21.0	~0.5	1.5	~90.1
			82	~2	6.1	
2	31.5	3.5	30.8	~0.7	3.5	~92.4
			81.5	~1.9	9.0	
3	94.0	5	93.7	~2.3	11.2	~93.3
			81.4	~2.0	9.9	
4	112		97.8			~84.7
			75.8	~2	~7	
5	107		97.8			~88.1
			79.3	~2	~7	

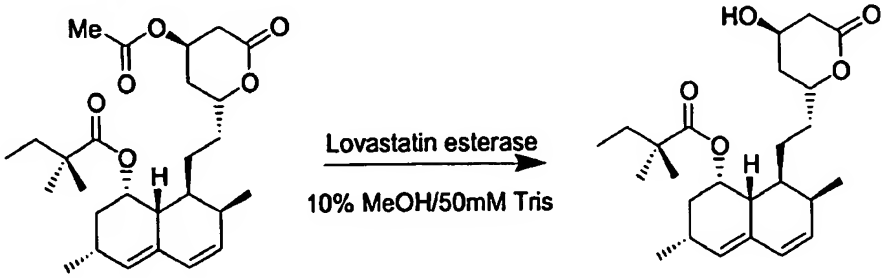
[1] Conditions: 4-Acetyllactone 10% w/v; $BF_3.OEt_2$ 8 mol%; 40°C; 5-9:1 DCM/MeCN

[2] Following precipitation from MeOH/water or MeOH alone

[3] Material in aqueous washes determined by HPLC assay against a working standard

[4] Remaining in mother liquors after concentration; determined by NMR assay against an internal standard

STEP 4: ENZYMATIC DEACETYLATION



4-Acetylsimvastatin **Simvastatin**

There are two significant hurdles to overcome in the enzymatic deacetylation of 4-acetyl simvastatin:

(i) the insolubility of both the starting material, 4-acetylsimvastatin, and the product, simvastatin, in aqueous solution,

(ii) the sensitivity of the 4-acetyl group, which rapidly undergoes elimination at pH >7.

Unlike the lovastatin hydrolysis reaction, the hydrolysis 4-acetyl simvastatin must be run close to pH 7 where increasing the solubility by opening the lactone ring is not possible.

Initial results for the hydrolysis of 4-acetylsimvastatin were very encouraging. Using BD12282 (the esterase gene cloned in E. coli), 10 mM substrate was hydrolyzed rapidly. Subsequent reactions at 200 mM indicated 91-93% conversion in 46 h; the 4-chloroacetyl derivative showed comparable conversion, while the 4-formyl derivative reacted completely in 24 h. While the 4-formyl derivative was an attractive substrate in terms of its solubility and reactivity, we were unable to develop an efficient synthesis of it. Similar results were obtained for all three derivatives when the reaction was carried out in a MTBE biphasic system.

A number of reaction parameters were examined using SEQ ID NO:4 (initially derived from *P. fluorescens*, it is an esterase gene in *P. fluorescens*). Starting the hydrolysis at pH 8 resulted in the formation of an unacceptable level of elimination product, while poor results were obtained using 5% dioxane as cosolvent or surfactants (0.1% Triton X-100 or Tween-20). While the rate of the reaction was considerably enhanced at 50°C, all reactions generally stopped at ~90% conversion as the reaction mixture became increasingly viscous.

For biphasic reactions at 50 mM substrate the use of MTBE, dibutyl ether or toluene as cosolvent worked well under these conditions, whereas the use of chlorinated solvents resulted in negligible activity.

It was possible to run the reaction at up to 300-400 mM if the hydrolysis was started at 50°C, pH 7 in the presence of 10% MeOH. After 5-6 h, as the reaction became very viscous, an equal volume of toluene was added to the reaction. Under these conditions almost complete conversion was observed with minimal elimination.

Up to this stage all enzymatic reactions had been run using 4-acetylsimvastatin that had been prepared from simvastatin. Preparing the substrate from the readily available simvastatin allowed us to carry out initial studies of the final enzymatic hydrolysis while the other steps of the synthesis were being developed.

Unfortunately, substrate which was initially prepared from lovastatin was variable in quality, depending on the Lewis acid catalyst used and the extent of purification. These materials resulted in a significant amount of variability in the results, and the initial good results for the enzymatic deacetylation were not reproducible.

The most recent results for the hydrolysis of 4-acetylsimvastatin to simvastatin are collected in Table 6. In this case all reactions have been run using 10%MeOH and the same batch of enzyme (SEQ ID NO:4-2).

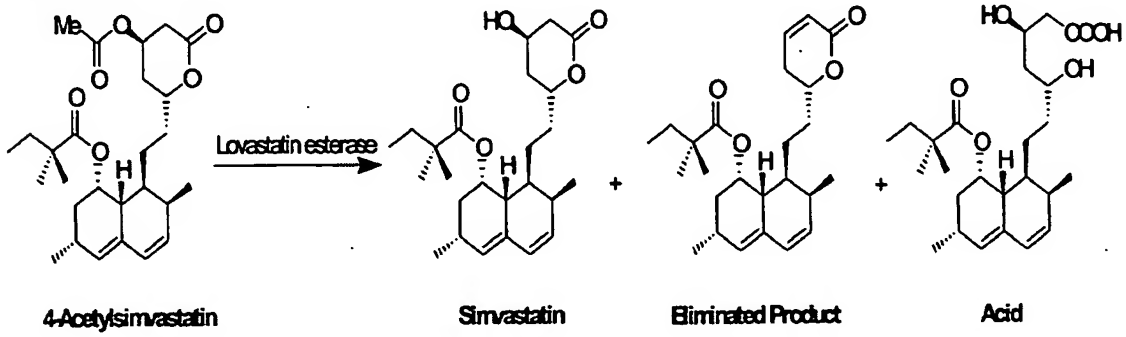


Table 6. Enzymatic Hydrolysis of 4-Acetylsimvastatin

Run	Batch	Scale g	mM	Temp °C	pH	Time h	Acid %	Simva %	4-Acsim %	Elimin %
1	2719-93	10	200	50	7.0	79	1.3	90.8	6.6	1.3
2	Synthetic	10	200	50	7.0	43	1.5	93.9	1.1	3.5
3	Synthetic	20	200	50	7.0	79	3.8	92.7	0	3.5
4	2719-95	20	200	50	7.0	45	3.0	95.5	0.5	1.0
5	"	5	100	50	7.0	45	1.4	95.3	2.7	0.6
6	"	5	200	50	7.5	33	2.5	94.8	1.6	1.1
7*	"	5	200	50	7.0	45	1.5	96.1	1.4	0.9
8	"	5	200	45	7.0	45	1.3	94.2	3.6	0.9
9	"	5	200	40	7.0	22		42.7	56.7	0.6
10	"	10	200	50	7.5	18	1.3	93.2	4.5	1.0
11	"	5	200	50	8.0	18	2.9	93.5	2.1	1.5
12	"	5	200	50	7.0	18	0.9	84.1	14.2	0.9

*Enzyme added in 4 portions over 24 h

The first two runs in Table 6 compare the hydrolysis of 4-acetylsimvastatin prepared from lovastatin (run1) with that prepared from simvastatin (run 2). At 200 mM, substrate 2719-93 was clearly inferior, requiring 79 h to reach 92% conversion compared to 43 h for the substrate prepared from simvastatin (run 2). On the other hand substrate 2719-95 (run 4) reached 98% conversion in 45 h, compared to 79 h for the synthetic substrate (run 3) at 200 mM. Substrate 2719-93 had shown low purity, being contaminated with residual 2,2-dimethylbutyric acid and giving consistently poor results. While no inhibitory effect had been observed in the presence of 2,2-dimethylbutyric acid at low conversions, it is possible that it might be responsible for a marked slowing down of the rate of hydrolysis at high conversions.

4-Acetylsimvastatin prepared from simvastatin performed poorly on a 20 g scale (cf., runs 2,3). While this result may reflect problems in stirring the larger scale reaction, this material reacted more slowly than substrate 2719-95 (run 4). While the eliminated product could possibly act as an irreversible inhibitor due to its potential to act as a Michael acceptor, no inhibitory effect was observed at low conversion when the reaction was run in the presence of the elimination product.

Recent results using substrate 2719-95 gave consistent results. The reaction gave similar results at 100 and 200 mM (runs 5,6) which may reflect the constant, low solubility of the starting material in the reaction mixture. At pH 7, higher conversions were observed at 50°C than at 40-45°C (run 7-9). Runs 10-12 indicate that the reaction is somewhat pH dependent, with higher conversions (94-96%) being observed at pH 7.5-8.0 compared to pH 7 (85%). Again this may reflect a higher solubility of the substrate under more basic conditions. However, the increase in conversion was accompanied by a slight increase in the level of simvastatin acid at higher pH. While higher pH increased the rate of the reaction it did not significantly increase the amount of elimination up to pH 8. Indeed all reactions showed <2 area% eliminated product, with the exception of runs 2,3; the starting 4-acetylsimvastatin for runs 2,3 was already contaminated with ~3.5% elimination product.

Further studies of the enzymatic reaction concentrated on attempts to shorten the reaction time by varying the reaction temperature and pH. The data in Table 7 indicate that reaction times can be shortened by operating at higher temperature, but the data may be

complicated by the effects of stirring different scale reactions (cf. Runs 13-16). However, increasing temperature and/or acid results in an increase in the amount of simvastatin acid formed, but in general did not result in a significant increase in elimination (the highest amount was observed at 60°C and pH 8 (Run 20)). Under the present lab scale workup, this simvastatin acid is lost in the aqueous stream. However workup conditions involving an acidic workup might relactonization to simvastatin with capture of some of this material.

Table 7. Hydrolysis of 4-Acetylsimvastatin: Effect of Temperature and pH

Run	Batch	Scale g	Temp °C	pH	Time	Acid %	Simva %	4-Acsimv %	Elim %
13	1 2719-95	5	55	7.5	18	2.3	95.0	1.6	1.1
14**	1 2958-8	10	55	7.5	36	3.5	95.0	0.7	0.9
15**	1 2958-8	10	55	7.5	36	3.7	93.5	1.8	0.9
16	1 4-38-1	40	55	7.5	41	5.5	91.3	1.7	1.4
17	1 4-38-1	20	55	7.5	41	4.9	92.9	0.8	1.4
18	1 4-38-1	5	60	7.5	41	5.2	93.0	0.6	1.2
19	1 4-38-1	5	55	8.0	15.5	2.9	94.4	1.3	1.3
20	2 4-38-1	5	60	8.0	15.5	9.0	81.3	6.6	3.1
21	2 2958-12	96.6	60	7.5	18	0.3 .2	39.7 2.1	59.5 .1	0.6 .4

2	5	9	1	1	
4	.1	1.4	.7	.8	

All reaction run at 200 mM

* - batch-wise addition of enzyme

** - duplicates

The latest experiment (Run 21) at 100 g scale was run at 60°C and pH 7.5, with a combination of magnetic and overhead stirring to efficiently mix the contents of the reaction flask. Under these conditions ~98% conversion of starting material was observed after 24 h.

Workup of the enzyme-catalyzed hydrolysis presented a challenge at the lab-bench scale. Filtration of the reaction mixture was very slow, presumably due to fouling of the filter by precipitated protein. Instead, centrifugation was a convenient method to separate the precipitated simvastatin from the bulk of the supernatant aqueous solution; most of the simvastatin acid is lost at this stage. The wet centrifuge pellet was then digested twice with CH_2Cl_2, the supernatant being decanted each time. The combined organic supernatant, which contained the bulk of the simvastatin product, was dried, filtered and the solvent exchanged for toluene. Addition of hexanes to this toluene solution and cooling resulted in the precipitation of simvastatin.

Even after digestion with CH_2Cl_2 the centrifuge pellet still contained a significant quantity of product; presumably the CH_2Cl_2 cannot efficiently access the wet centrifuge pellet and extract out the entrained product.

In a recent modification (Run 4; Table 8), the centrifuge pellet was treated with acetone and Celite and then filtered. The Celite pad could then be easily extracted with CH_2Cl_2. The combined aqueous acetone and CH_2Cl_2 washings were then dried and the solvent exchanged for toluene. Addition of hexanes resulted in the immediate precipitation of simvastatin which was filtered and dried. Cooling the mother liquor to -20°C resulted in the isolation of a second crop; the yield data in Table 8 (Figure 4) are for combined 1st and 2nd crops.

The invention provides novel practical routes for generating Simvastatin starting from Lovastatin. In alternative aspects of the invention, salient features of the route comprise:

i. The use of a novel lovastatin esterase which can remove the 2-methylbutyrate side-chain with 99% conversion in ~48 h at a substrate loading of 0.5M at 35°C and pH 9.5. The possibility of significantly increasing the rate of reaction by increasing the reaction temperature exists. The demonstration of a 1-pot lactonization/acetylation which converts crude triol acid to 4-acetyllactone. Overall yields of 80% from lovastatin have been routinely achieved with a further 8-10% of potential product remaining in mother liquors.

ii. The discovery of novel and mild conditions for the introduction of the simvastatin side-chain using $BF_3.OEt_2$ catalyzed acylation with dimethylbutyric anhydride. The reaction has been run consistently at ~100 g scale at 10% substrate loading, providing 4-acetylsimvastatin in ~80% yield. A further 8-10% of potential product remains in the reaction residues.

iii. The final step uses the same lovastatin esterase as used in the first step to remove a sensitive acetyl group to yield simvastatin. This reaction has been run on a 20-100 g scale at 9% w/v substrate loading showing 98% in 24-48 h.

iv. A crude cost analysis has been undertaken and has been reported separately. Assuming yields of 80%, 90% and 80% for each step, and discounting Aldrich catalog prices, the chemical cost contribution for this process was estimated at $130/kg simvastatin.

A number of embodiments of the invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention. Accordingly, other embodiments are within the scope of the following claims.

WHAT IS CLAIMED IS:

1. A method for the preparation of simvastatin comprising (i) at least one protocol as set forth in Appendix A or Appendix B, or, (ii) a process comprising steps 1 through 5, wherein

step 1 comprises enzymatic hydrolysis of lovastatin, lovastatin acid or a salt of lovastatin acid to form the triol acid;

step 2 comprises heating the triol acid or stirring in the presence of acid to form the diol lactone;

step 3 comprise protection of the 4'-OH on the lactone ring by regioselective acylation either chemically or enzymatically;

step 4 comprises acylation of the hydroxyl at the 8-position carried out chemically or enzymatically using a hydrolase; and

step 5 comprises selective removal of the acyl protecting group at the 4' position either chemically or enzymatically, thereby yielding simvastatin.

2. The method of claim 1, wherein at least one step is performed in a reaction vessel.

3. The method of claim 1, wherein at least one step is performed in a cell extract.

4. The method of claim 1, wherein at least one step is performed in a whole cell.

5. The method of claim 1, wherein an ammonium salt of simvastatin is formed.

6. The method of claim 1, further comprising crystallization of the simvastatin.

7. The method of claim 6, further comprising re-crystallization of the simvastatin.

8. The method of claim 6, further comprising relactonization to provide simvastatin with a desired purity.

9. The method of claim 1, wherein at least one enzymatic reaction is carried out by a hydrolase encoded by a nucleic acid having at least 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more, or complete (100%) sequence identity to SEQ ID NO:1, or enzymatically active fragments thereof..

10. The method of claim 1, wherein at least one enzymatic reaction is carried out by a hydrolase encoded by a nucleic acid having at least 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more, or complete (100%) sequence identity to SEQ ID NO:3, or enzymatically active fragments thereof..

11. The method of claim 1, wherein at least one enzymatic reaction is carried out by a hydrolase encoded by a nucleic acid having at least 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more, or complete (100%) sequence identity to SEQ ID NO:5, or enzymatically active fragments thereof..

12. The method of claim 1, wherein at least one enzymatic reaction is carried out by a hydrolase having a sequence at least about 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more, or complete (100%) sequence identity to SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6, or enzymatically active fragments thereof.

13. The method of claim 1, wherein the protocol to prepare simvastatin comprises enzymatic or chemical hydrolysis of lovastatin to make a triol acid, followed by lactonization of the triol acid to make a diol lactone, followed by chemical or enzymatic acylation of the diol lactone to make a 4-acetyl lactone, followed by chemical or enzymatic acylation of the 4-acetyl lactone to make a 4-acetyl-simvastatin, followed by enzymatic hydrolysis of the 4-acetyl-simvastatin to make simvastatin.

14. A method for preparing 4-acetyl lactone comprising enzymatic hydrolysis of lovastatin to make a triol acid, followed by lactonization of the triol acid to make a diol lactone, followed by chemical or enzymatic acylation of the diol lactone to make 4-acetyl lactone.

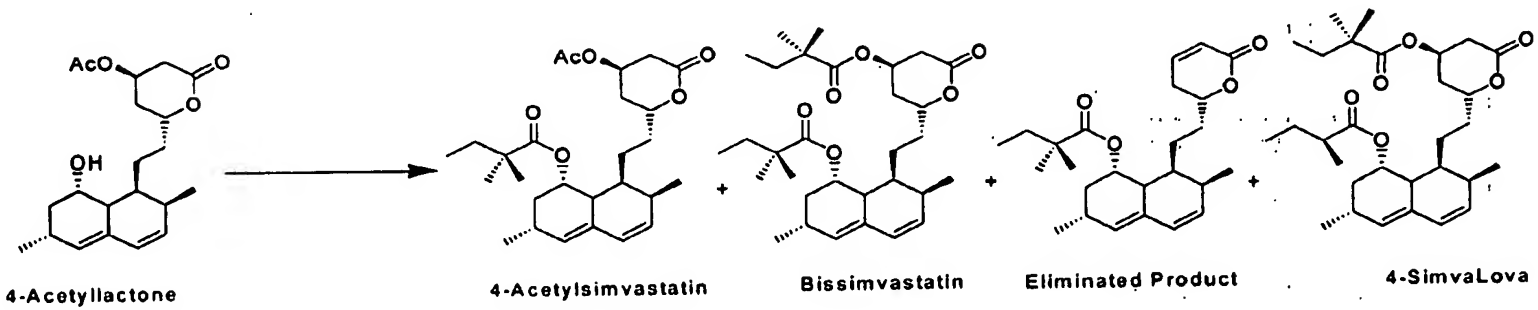
15. A method for preparing 4-acetyl-simvastatin comprising enzymatic hydrolysis of lovastatin to make a triol acid, followed by lactonization of the triol acid to make a diol lactone, followed by chemical or enzymatic acylation of the diol lactone to make 4-acetyl lactone.

ABSTRACT

METHODS FOR MAKING SIMVASTATIN AND INTERMEDIATES

The invention provides synthetic chemical and chemoenzymatic methods of producing simvastatin and various intermediates. In one aspect, enzymes such as hydrolases are used in the methods of the invention.

Figure 1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O
O
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+
O
O
O
Eliminated Product
+
O
O
O
O
O
4-SimvaLova

Figure 2

Table 3. Survey of conditions for the acylation of 4-acetyllactone

Run	BF_3 .OEt_2 mol%	DCM:MeCN	Time h	Mass[1] balance %	4-Ac Sim %	SM %	DiOAc %	4-Ac Lova %	Elimin %	4-Sim-Lova %	BisSim[3] %
1	8	1:1	0.5	94.8	90.8	1.2	2.8	1.1	0.5	2.7	0.9
2	8 (Old)	1:1	5.3	85.3	90.7	0.6	3.3	1.2	0.9	2.5	0.8
3	8	9:1	6.5	89.7	91.1	0.6	2.7	1.1	0.8	2.7	1.0
4	4	1:1	17.5	78.9	36.3	55.9	3.9	1.0	1.3	1.0	0.6
5	8	5:1	1.1	92.8	96.9	0.3	0.9	0.5	0.4		1.0
6	8 (Stock)	5:1	4.5	87.1	96.1	0.5	1.0	0.5	0.7		1.2

[1]Crude weight yield. [2]Lovastatin with 2,2-dimethylbutyrate at the 4-position. [3] 2,2-Dimethylbutyrate at the 4,8-positions.

Figure 3

Table 4. Purification of 4-Acetylsimvastatin by precipitation from MeOH

	DCE:MeCN	Wt yield %	4-AcSim %	SM %	DIOAc %	4-Ac Lova %	Elimin %	BisSim %
Crude product	5:1	112[1]	96.4	1.0	0.9	0.3	0.7	0.7
After precipitation		93	98.5	0.2	0.9	0.2	0.2	

[1] Contaminated with 2,2-dimethylbutyic acid

Figure 4

Table 8. Isolation of Simvastatin

Run	Scale g	Batch	Theoret Yield g	Isolated Yield g	Yield %	Acid %	Simva %	4-Acsim %	Elimin %	Lova %
1	50	Pooled: various	45.4	33.8	74.4	0.2	96.7	1.7	1.2	0.2
2	45	Pooled same batch	40.9	27.7	67.7	0.3	97.8	1.3	0.3	0.4
3	25	Pooled Same batch	22.7	17.2	75.7	0.3	97.7	1.3	0.3	0.4
4	96.6	Single batch	87.8	73.4	83.6	0.7	97.5	1.0	0.6	0.2

SEQUENCE LISTING

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Levin, Michael

<120> METHODS FOR MAKING SIMVASTATIN AND INTERMEDIATES

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<213> Unkown

<220>
<223> Environmental

<220>
<221> SIGNAL
<222> (1)...(24)

<400> 2
Met Ser Leu Cys Val Ile Arg Phe Ile Ala Gly Thr Leu Val Leu Val
1 5 10 15

Ala Ser Val Glu Ser Ala Val Ala Gln Gln Ala Cys Ala Asp Leu Met
20 25 30

Gly Leu Glu Leu Pro Tyr Thr Thr Ile Thr Ser Ala Ala Val Ala Thr
35 40 45

Glu Gly Pro Ile Pro Gln Pro Ala Ile Phe Gly Ser Thr Asp Pro Ile
50 55 60

Val Ala Pro Glu Arg Cys Glu Val Arg Ala Val Thr Arg Pro Thr Lys
65 70 75 80

Asp Ser Glu Ile Arg Ile Glu Leu Trp Leu Pro Leu Ser Gly Trp Asn
85 90 95

Gly Lys Tyr Leu Gln Ile Gly Ser Gly Gly Trp Ala Gly Ser Ile Asn
100 105 110

Arg Thr Gly Leu Ile Gly Pro Leu Gln Arg Gly Tyr Ala Val Ala Ala
115 120 125

Thr Asp Asn Gly His Ile Ser Glu Gly Leu Val Pro Asp Ala Ser Trp
130 135 140

Ala Ile Gly His Pro Gln Lys Leu Ile Asp Phe Gly Tyr Arg Ala Val
145 150 155 160

His Glu Thr Ser Val Gln Ala Lys Ala Ile Leu Arg Ala Tyr Phe Gly
165 170 175

Arg Gly Gln Asp Leu Ser Tyr Phe Ser Gly Cys Ser Asn Gly Gly Arg
180 185 190

Glu Ala Leu Met Glu Ala Gln Arg Tyr Pro Glu Asp Phe Glu Gly Ile
195 200 205

Ile Ala Gly Ala Pro Ala Asn Asn Trp Ser Arg Leu Phe Thr Gly Phe
210 215 220

Val Trp Asn Glu Arg Ala Leu Ala Asp Asp Pro Ile Pro Pro Ala Lys
225 230 235 240

Leu Thr Ala Ile Gln Ala Ala Ala Ile Ala Ala Cys Asp Thr Leu Asp
245 250 255

Gly Val Glu Asp Gly Leu Ile Glu Asn Pro Arg Ala Cys Ser Phe Asp
260 265 270

Pro Arg Ser Met Val Cys Thr Ala Asp Asp Ala Ser Asp Cys Leu Thr
275 280 285

Glu Gly Gln Val Ala Thr Leu His Arg Ile Tyr Ser Gly Pro Thr Asn
290 295 300

Pro Arg Thr Gly Glu Arg Ile Phe Pro Gly Tyr Pro Met Gly Thr Glu
305 310 315 320

Ala Val Pro Gly Gly Trp Val Pro Trp Ile Val Ser Ala Ser Ser Glu
325 330 335

Val Pro Ser Ile Gln Ala Ser Phe Gly Asn Ser Tyr Tyr Gly His Ala
340 345 350

Val Phe Glu Gln Ser Asn Trp Asp Phe Arg Thr Leu Asp Phe Asp Gln
355 360 365

Asp Val Ala Phe Gly Asp Ala Lys Ala Gly Pro Val Leu Asn Ala Thr
370 375 380

Asn Pro Asp Leu Arg Ser Phe Arg Ala Asn Gly Gly Lys Leu Ile Gln
385 390 395 400

Tyr His Gly Trp Gly Asp Ala Ala Ile Thr Ala Phe Ser Ser Ile Asp
405 410 415

Tyr Tyr Glu Asn Val Arg Ala Phe Leu Asp Arg Phe Pro Asp Pro Arg
420 425 430

Ser Glu Asn Thr Asp Ile Asp Gly Phe Tyr Arg Leu Phe Leu Val Pro
435 440 445

Gly Met Gly His Cys Ser Gly Gly Ile Gly Pro Ser Ser Phe Gly Asn
450 455 460

Gly Phe Arg Ser Ala Arg Thr Asp Ala Glu His Asp Leu Leu Ser Ala
465 470 475 480

Leu Glu Ala Trp Val Glu Arg Asp Thr Ala Pro Glu Arg Leu Ile Gly
485 490 495

Thr Gly Thr Ala Val Gly Asp Pro Thr Ala Thr Leu Thr Arg Pro Leu
500 505 510

Cys Pro Tyr Pro Arg Thr Ala Arg Tyr Leu Gly Ser Gly Asn Ser Asn
515 520 525

Asp Ala Ala Asn Phe Glu Cys Ala Leu Pro Ala Gly Val Gln
530 535 540

<210> 3
<211> 1209
<212> DNA
<213> Unknown

<220>
<223> Environmental

<400> 3
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aatttgcgtg agcgcggcga agtaggagcg tccgtttgcg tcacgttgca cggcgaaacc 120

gtagtggact tgtggggcgg catggcgcgt gccgacactc agacgccatg gacggcggag 180

acggtcagta ttgttttttc ctccaccaaa ggcgcaacgg cactctgcgc ccatatgctg 240

gcgtcacgcg gccaactgga tcttgatgca ccagtcgcca cctactggcc ggaatttgcc 300

caagccggca aagctcgcat cccggtgaaa atgctcttga accatcaagc tggtctccct 360

gccgtacgga caccgctgcc ccagggtgcc tacgctgact gggaactgat ggtcaatacg 420

ttggccaagg aagagccgtt ttgggaacct ggcacccgca acggctatca tgcgctcacc 480

atggggtggc tggtgggaga agtggtgcga cgtgtctctg gtaagtcgct tgggacattc 540

ttccaagagg agatcgccag gccgttgggg ttagatttct ggattggctt accagcagag 600

caagaggcac gggtcgcgcc gatgatcgcg gcggagcctg atccgcaaag cctcttcttc 660

caagaggtcg cgaagcctgg ggccttacag tcgctcgtac tccttaactc cggcggctat 720

atgggtgctc agcctgagta tgactcgcgg gcggcgcatg cggccgagat tggtgcagcc 780

ggtggtatca ccaacgcacg cggcctggca ggcatgtacg caccactggc ctgcggaggc 840

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tctgcgactg ggagagatgc cgtgctcatg atgccaaccc ggtttgccct gggcttcatg 960

aagtccatgg acaaccgccg ggagcctgct ggcgtgcagg acagcgcgct ctttggggag 1020

gaggcttttg gccatgtggg ggccgggggt tcgtttggtt ttgccgatcc caaagcagga 1080

atgtcctttg gctataccat gaaccgaatg gggctgggag ccgggctcaa cccgcggggg 1140

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tggacctga 1209

<210> 4
<211> 402
<212> PRT
<213> Unkown

<220>
<223> Environmental

<220>
<221> DOMAIN
<222> (18)...(386)
<223> Beta-lactamase

<400> 4
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Glu Phe Glu Arg Asn Leu Arg Glu Arg Gly Glu Val Gly Ala Ser Val
20 25 30

Cys Val Thr Leu His Gly Glu Thr Val Val Asp Leu Trp Gly Gly Met
35 40 45

Ala Arg Ala Asp Thr Gln Thr Pro Trp Thr Ala Glu Thr Val Ser Ile
50 55 60

Val Phe Ser Ser Thr Lys Gly Ala Thr Ala Leu Cys Ala His Met Leu
65 70 75 80

Ala Ser Arg Gly Gln Leu Asp Leu Asp Ala Pro Val Ala Thr Tyr Trp
85 90 95

Pro Glu Phe Ala Gln Ala Gly Lys Ala Arg Ile Pro Val Lys Met Leu
100 105 110

Leu Asn His Gln Ala Gly Leu Pro Ala Val Arg Thr Pro Leu Pro Gln
115 120 125

Gly Ala Tyr Ala Asp Trp Glu Leu Met Val Asn Thr Leu Ala Lys Glu
130 135 140

Glu Pro Phe Trp Glu Pro Gly Thr Arg Asn Gly Tyr His Ala Leu Thr
145 150 155 160

Met Gly Trp Leu Val Gly Glu Val Val Arg Arg Val Ser Gly Lys Ser
165 170 175

Leu Gly Thr Phe Phe Gln Glu Glu Ile Ala Arg Pro Leu Gly Leu Asp
180 185 190

Phe Trp Ile Gly Leu Pro Ala Glu Gln Glu Ala Arg Val Ala Pro Met
195 200 205

Ile Ala Ala Glu Pro Asp Pro Gln Ser Leu Phe Phe Gln Glu Val Ala
210 215 220

Lys Pro Gly Ala Leu Gln Ser Leu Val Leu Leu Asn Ser Gly Gly Tyr
225 230 235 240

Met Gly Ala Gln Pro Glu Tyr Asp Ser Arg Ala Ala His Ala Ala Glu
245 250 255

Ile Gly Ala Ala Gly Gly Ile Thr Asn Ala Arg Gly Leu Ala Gly Met
260 265 270

Tyr Ala Pro Leu Ala Cys Gly Gly Lys Leu Lys Gly Val Glu Leu Val
275 280 285

Ser Pro Asp Met Leu Ala Arg Met Ser Arg Val Ala Ser Ala Thr Gly
290 295 300

Arg Asp Ala Val Leu Met Met Pro Thr Arg Phe Ala Leu Gly Phe Met
305 310 315 320

Lys Ser Met Asp Asn Arg Arg Glu Pro Ala Gly Val Gln Asp Ser Ala
325 330 335

Leu Phe Gly Glu Glu Ala Phe Gly His Val Gly Ala Gly Gly Ser Phe
340 345 350

Gly Phe Ala Asp Pro Lys Ala Gly Met Ser Phe Gly Tyr Thr Met Asn
355 360 365

Arg Met Gly Leu Gly Ala Gly Leu Asn Pro Arg Gly Gln Ser Leu Val
370 375 380

Asp Ala Thr Tyr Arg Ser Leu Gly Tyr Gln Ser Asp Ala Ser Gly Ala
385 390 395 400

Trp Thr

<210> 5
<211> 1578
<212> DNA
<213> Unknown

<220>
<223> Environmental

<400> 5
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cttcccaaca cgacgatcac ctcggcccag accgtcacca ccggatcgtt aacgcccccg 180

ggctcgacga atccgatcac cgacctgcct cctttctgcc gtgtcacagg cgccatcgcc 240

ccgacgagcg agtcgcacat cctcttcgag gtctggctgc cgctggataa atggaacggc 300

aagttcgccg gcgtgggcaa cggcggctgg gccggcatca tctccttcgg cgccctcgga 360

agccagctca agcgcggcta cgcgaccgcc tccacgaata cgggtcacga agcggcgccg 420

gggatgaacg cagccaggtt tgcgttcgag aagccggagc agcttatcga cttcgcctat 480

cgctcccagc acgagacggc cctgaaagcg aaggcgctgg ttcaggcttt ctacgggaag 540

ccgccggaac actcctattt catcgggtgc tcatcgggtg ggtaccaggg cctgatggag 600

gcccaacgat ttccggccga ctacgacggg atcgtcgccg gtatgccggc gaacaactgg 660

acacggctga tggccggcga cttggacgcg atccttgccg tctccgtaga tcctgccagc 720

caccttcccg tctccgcatt gggtctgttg tatcgctcgg tgctcgctgc ctgcgacggc 780

atcgacggtg ttgtagacgg tgttctggag gatccgcgcc gatgccggtt cgacccggcc 840

gtgttgatgt gcaaggcgga tcagaatccc gatggctgcc ttacgccggc tcaggtggaa 900

gcggcacggc gcatatacgg cggtctgaag gatcccaaga ccggcgctca gctctatccg 960

gggctggcgc cgggaagcga gccgttctgg ccgcaccgca atccggcgaa tccgttccct 1020

attccgatcg cgcactacaa gtggctcgtc tttgccgatc caaactggga ttggagaaca 1080

ttcaagttca cggatccggc ggactaccag gctttcctca atgcggaagc cacgtatgcc 1140

cctactctca atgcgaccaa tccggacctc cgggagttca gccggcgcgg cggcaggttg 1200

attcagtacc atggctggaa cgatcagctg attgccccgc aaaacagcat cgactattac 1260

gagagcgtcc tttcgttctt cgggtccggc aaacaggatc gagcgcagac cgtgcgcgag 1320

gttcagagct tctaccggct gttcatggcg ccgggtatgg ctcactgtgg aggcggtaca 1380

ggtccgaact catttgacat gctggatgcc ctcgagaagt gggtggaagg cgggatagcg 1440

ccggaacgag tccttgcgac gcgttccata aacggcgtag tcgaccggct gcgcccgctc 1500

tgtccatatc cgcaggtcgc cgtgtacaag ggtcatgggg atacaaacga cgccgcgaac 1560

ttcgtctgtc gcgattag 1578

<210> 6
<211> 525
<212> PRT
<213> Unkown

<220>
<223> Environmental

<220>
<221> SIGNAL
<222> (1)...(25)

<400> 6
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1 5 10 15

Leu Leu Leu Thr Thr Arg Val Ser Ala Gln Ile Val Pro Ala Met Glu
20 25 30

Cys Ala Asp Leu Ala Asn Gln Gln Leu Pro Asn Thr Thr Ile Thr Ser
35 40 45

Ala Gln Thr Val Thr Thr Gly Ser Leu Thr Pro Pro Gly Ser Thr Asn
50 55 60

Pro Ile Thr Asp Leu Pro Pro Phe Cys Arg Val Thr Gly Ala Ile Ala
65 70 75 80

Pro Thr Ser Glu Ser His Ile Leu Phe Glu Val Trp Leu Pro Leu Asp
85 90 95

Lys Trp Asn Gly Lys Phe Ala Gly Val Gly Asn Gly Gly Trp Ala Gly
100 105 110

Ile Ile Ser Phe Gly Ala Leu Gly Ser Gln Leu Lys Arg Gly Tyr Ala

115 120 125

Thr Ala Ser Thr Asn Thr Gly His Glu Ala Ala Pro Gly Met Asn Ala
130 135 140

Ala Arg Phe Ala Phe Glu Lys Pro Glu Gln Leu Ile Asp Phe Ala Tyr
145 150 155 160

Arg Ser Gln His Glu Thr Ala Leu Lys Ala Lys Ala Leu Val Gln Ala
165 170 175

Phe Tyr Gly Lys Pro Pro Glu His Ser Tyr Phe Ile Gly Cys Ser Ser
180 185 190

Gly Gly Tyr Gln Gly Leu Met Glu Ala Gln Arg Phe Pro Ala Asp Tyr
195 200 205

Asp Gly Ile Val Ala Gly Met Pro Ala Asn Asn Trp Thr Arg Leu Met
210 215 220

Ala Gly Asp Leu Asp Ala Ile Leu Ala Val Ser Val Asp Pro Ala Ser
225 230 235 240

His Leu Pro Val Ser Ala Leu Gly Leu Leu Tyr Arg Ser Val Leu Ala
245 250 255

Ala Cys Asp Gly Ile Asp Gly Val Val Asp Gly Val Leu Glu Asp Pro
260 265 270

Arg Arg Cys Arg Phe Asp Pro Ala Val Leu Met Cys Lys Ala Asp Gln
275 280 285

Asn Pro Asp Gly Cys Leu Thr Pro Ala Gln Val Glu Ala Ala Arg Arg
290 295 300

Ile Tyr Gly Gly Leu Lys Asp Pro Lys Thr Gly Ala Gln Leu Tyr Pro
305 310 315 320

Gly Leu Ala Pro Gly Ser Glu Pro Phe Trp Pro His Arg Asn Pro Ala
325 330 335

Asn Pro Phe Pro Ile Pro Ile Ala His Tyr Lys Trp Leu Val Phe Ala
340 345 350

Asp Pro Asn Trp Asp Trp Arg Thr Phe Lys Phe Thr Asp Pro Ala Asp
355 360 365

Tyr Gln Ala Phe Leu Asn Ala Glu Ala Thr Tyr Ala Pro Thr Leu Asn

370 375 380

Ala Thr Asn Pro Asp Leu Arg Glu Phe Ser Arg Arg Gly Gly Arg Leu
385 390 395 400

Ile Gln Tyr His Gly Trp Asn Asp Gln Leu Ile Ala Pro Gln Asn Ser
405 410 415

Ile Asp Tyr Tyr Glu Ser Val Leu Ser Phe Phe Gly Ser Gly Lys Gln
420 425 430

Asp Arg Ala Gln Thr Val Arg Glu Val Gln Ser Phe Tyr Arg Leu Phe
435 440 445

Met Ala Pro Gly Met Ala His Cys Gly Gly Gly Thr Gly Pro Asn Ser
450 455 460

Phe Asp Met Leu Asp Ala Leu Glu Lys Trp Val Glu Gly Gly Ile Ala
465 470 475 480

Pro Glu Arg Val Leu Ala Thr Arg Ser Ile Asn Gly Val Val Asp Arg
485 490 495

Leu Arg Pro Leu Cys Pro Tyr Pro Gln Val Ala Val Tyr Lys Gly His
500 505 510

Gly Asp Thr Asn Asp Ala Ala Asn Phe Val Cys Arg Asp
515 520 525

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